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a corporation organized under the laws of the State of Delaware
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La soussignée

PRINCIPAUTÉ DE MONACO
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EP No. 0,937,098 (Appln. No. 96923288.3)

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X Name: Alan Hammond

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It is hereby certified that a European patent has been granted in respect of the invention described in the annexed patent specification for the Contracting States designated in the specification.

Il est certifié qu'un brevet européen a été délivré pour l'invention décrite dans le fascicule de brevet ci-joint, pour les Etats contractants désignés dans le fascicule de brevet.

Europäisches Patent Nr. **European Patent No.** **Brevet européen n°**

0937098

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(54) **RECOMBINATIONAL CLONING IN VITRO USING ENGINEERED RECOMBINATION SITES**

REKOMBINATORISCHE KLONIERUNG IN VITRO UNTER VERWENDUNG GENMANIPULIERTER REKOMBINATIONSORTE

CLONAGE DE RECOMBINAISON IN VITRO AU MOYEN DE SITES RECOMBINAISON OBTENUS PAR GENIE GENETIQUE

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- **SCHLAKE T ET AL: "USE OF MUTATED FLP RECOGNITION TARGET (FRT) SITES FOR THE EXCHANGE OF EXPRESSION CASSETTES AT DEFINED CHROMOSOMAL LOCI" BIOCHEMISTRY, vol. 33, no. 43, 1 November 1994 (1994-11-01), pages 12746-12751, XP000616165 ISSN: 0006-2960**
- **HASAN N ET AL: "ESCHERICHIA COLI GENOME TARGETING, I. CRE-LOX-MEDIATED IN VITRO GENERATION OF ORI-PLASMIDS AND THEIR IN VIVO CHROMOSOMAL INTEGRATION AND RETRIEVAL" GENE, vol. 150, no. 1, 2 December 1994 (1994-12-02), pages 51-56, XP002916292 ISSN: 0378-1119**
- **SNAITH M R ET AL: "MULTIPLE CLONING SITES CARRYING LOXP AND FRT RECOGNITION SITES FOR THE CRE AND FLP SITE-SPECIFIC RECOMBINASES" GENE, vol. 166, no. 1, 1 December 1995 (1995-12-01), page 173/174 XP002061817 ISSN: 0378-1119**
- **BIOTECHNIQUES, June 1994, Vol. 16, No. 6, BRUNELLI et al., "Lambda/Plasmid Vector Construction by in Vivo Cre/Lox-Mediated Recombination", pages 1060 and 1062-1064.**

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between replicons. Transposons are structurally variable, being described as simple or compound, but typically encode the recombinase gene flanked by DNA sequences organized in inverted orientations. Integration of transposons can be random or highly specific. Representatives such as Tn7, which are highly site-specific, have been applied to the *in vivo* movement of DNA segments between replicons (Lucklow *et al.*, *J. Virol. 67*:4566-4579 (1993)).

[0015] Devine and Boeke *Nucl. Acids Res. 22*:3765-3772 (1994), discloses the construction of artificial transposons for the insertion of DNA segments, *in vitro,* into recipient DNA molecules. The system makes use of the integrase of yeast TY1 virus-like particles. The DNA segment of interest is cloned, using standard methods, between the ends of the transposon-like element TY1. In the presence of the TY1 integrase, the resulting element integrates randomly into a second target DNA molecule.

[0016] ***DNA cloning.*** The cloning of DNA segments currently occurs as a daily routine in many research labs and as a prerequisite step in many genetic analyses. The purpose of these clonings is various, however, two general purposes can be considered: (1) the initial cloning of DNA from large DNA or RNA segments (chromosomes, YACs, PCR fragments, mRNA, etc.), done in a relative handful of known vectors such as pUC, pGem, pBlueScript, and (2) the subcloning of these DNA segments into specialized vectors for functional analysis. A great deal of time and effort is expended both in the initial cloning of DNA segments and in the transfer of DNA segments from the initial cloning vectors to the more specialized vectors. This transfer is called subcloning.

[0017] The basic methods for cloning have been known for many years and have changed little during that time. A typical cloning protocol is as follows:

(1) digest the DNA of interest with one or two restriction enzymes;
(2) gel purify the DNA segment of interest when known;
(3) prepare the vector by cutting with appropriate restriction enzymes, treating with alkaline phosphatase, gel purify etc., as appropriate;
(4) ligate the DNA segment to vector, with appropriate controls to estimate background of uncut and self-ligated vector;
(5) introduce the resulting vector into an *E. coli* host cell;
(6) pick selected colonies and grow small cultures overnight;
(7) make DNA minipreps; and
(8) analyze the isolated plasmid on agarose gels (often after diagnostic restriction enzyme digestions) or by PCR.

[0018] The specialized vectors used for subcloning DNA segments are functionally diverse. These include but are not limited to: vectors for expressing genes in various organisms; for regulating gene expression; for providing tags to aid in protein purification or to allow tracking of proteins in cells; for modifying the cloned DNA segment (*e.g.*, generating deletions); for the synthesis of probes (*e.g.*, riboprobes); for the preparation of templates for DNA sequencing; for the identification of protein coding regions; for the fusion of various protein-coding regions; to provide large amounts of the DNA of interest, *etc.* It is common that a particular investigation will involve subcloning the DNA segment of interest into several different specialized vectors.

[0019] As known in the art, simple subclonings can be done in one day *(e.g.,* the DNA segment is not large and the restriction sites are compatible with those of the subcloning vector). However, many other subclonings can take several weeks, especially those involving unknown sequences, long fragments, toxic genes, unsuitable placement of restriction sites, high backgrounds, impure enzymes, *etc.* Subcloning DNA fragments is thus often viewed as a chore to be done as few times as possible.

[0020] Several methods for facilitating the cloning of DNA segments have been described, *e.g.,* as in the following references.

[0021] Ferguson, J., *et al. Gene 16*:191 (1981), discloses a family of vectors for subcloning fragments of yeast DNA. The vectors encode kanamycin resistance. Clones of longer yeast DNA segments can be partially digested and ligated into the subcloning vectors. If the original cloning vector conveys resistance to ampicillin, no purification is necessary prior to transformation, since the selection will be for kanamycin.

[0022] Hashimoto-Gotoh, T., *et al. Gene 41*:125 (1986), discloses a subcloning vector with unique cloning sites within a streptomycin sensitivity gene; in a streptomycin-resistant host, only plasmids with inserts or deletions in the dominant sensitivity gene will survive streptomycin selection.

[0023] Accordingly, traditional subcloning methods, using restriction enzymes and ligase, are time consuming and relatively unreliable. Considerable labor is expended, and if two or more days later the desired subclone can not be found among the candidate plasmids, the entire process must then be repeated with alternative conditions attempted. Although site specific recombinases have been used to recombine DNA *in vivo,* the successful use of such enzymes in *vitro* was expected to suffer from several problems. For example, the site specificities and efficiencies were expected to differ *in vitro;* topologically-linked products were expected; and the topology of the DNA substrates and recombination proteins was expected to differ significantly in *vitro (see, e.g.,* Adams *et al, J. Mol. Biol. 226*:661-73 (1992)). Reactions

constituting a functional Selectable marker when recombined across the first or second recombination site with another inactive fragment of at least one Selectable marker.

[0031] The present recombinational cloning method possesses several advantages over previous *in vivo* methods. Since single molecules of recombination products can be introduced into a biological host, propagation of the desired Product DNA in the absence of other DNA molecules (*e.g.*, starting molecules, intermediates, and by-products) is more readily realized. Reaction conditions can be freely adjusted *in vitro* to optimize enzyme activities DNA molecules can be incompatible with the desired biological host (*e.g.*, YACs, genomic DNA, etc.), can be used. Recombination proteins from diverse sources can be employed, together or sequentially.

Brief Description of the Figures

[0032] **Figure 1** depicts one general method of the present invention, wherein the starting (parent) DNA molecules can be circular or linear. The goal is to exchange the new subcloning vector D for the original cloning vector B. It is desirable in one embodiment to select for AD and against all the other molecules, including the Cointegrate. The square and circle are sites of recombination: *e.g., loxP* sites, *att* sites, *etc.* For example, segment D can contain expression signals, new drug markers, new origins of replication, or specialized functions for mapping or sequencing DNA.

[0033] **Figure 2A** depicts an *in vitro* method of recombining an Insert Donor plasmid (here, pEZC705) with a Vector Donor plasmid (here, pEZC726), and obtaining Product DNA and Byproduct daughter molecules. The two recombination sites are *attP* and *loxP* on the Vector Donor. On one segment defined by these sites is a kanamycin resistance gene whose promoter has been replaced by the *tetOP* operator/promoter from transposon *Tn*10. *See* Sizemore *et al., Nucl. Acids Res. 18*(10):2875 (1990). In the absence of tet repressor protein, *E. coli* RNA polymerase transcribes the kanamycin resistance gene from the *tetOP.* If tet repressor is present, it binds to *tetOP* and blocks transcription of the kanamycin resistance gene. The other segment of pEZC726 has the *tet* repressor gene expressed by a constitutive promoter. Thus cells transformed by pEZC726 are resistant to chloramphenicol, because of the chloramphenicol acetyl transferase gene on the same segment as *tetR,* but are sensitive to kanamycin. The recombinase-mediated reactions result in separation of the *tetR* gene from the regulated kanamycin resistance gene. This separation results in kanamycin resistance in cells receiving only the desired recombination products. The first recombination reaction is driven by the addition of the recombinase called Integrase. The second recombination reaction is driven by adding the recombinase Cre to the Cointegrate (here, pEZC7 Cointegrate).

[0034] **Figure 2B** depicts a restriction map of pEZC705.

[0035] **Figure 2C** depicts a restriction map of pEZC726.

[0036] **Figure 2D** depicts a restriction map of pEZC7 Cointegrate.

[0037] **Figure 2E** depicts a restriction map of Intprod.

[0038] **Figure 2F** depicts a restriction map of Intbypro.

[0039] **Figure 3A** depicts an *in vitro* method of recombining an Insert Donor plasmid (here, pEZC602) with a Vector Donor plasmid (here, pEZC629), and obtaining Product (here, EZC6prod) and Byproduct (here, EZC6Bypr) daughter molecules. The two recombination sites are *loxP* and *loxP 511.* One segment of pEZC629 defined by these sites is a kanamycin resistance gene whose promoter has been replaced by the *tetOP* operator/promoter from transposon *Tn*10. In the absence of tet repressor protein, *E. coli* RNA polymerase transcribes the kanamycin resistance gene from the *tetOP.* If tet repressor is present, it binds to *tetOP* and blocks transcription of the kanamycin resistance gene. The other segment of pEZC629 has the *tet* repressor gene expressed by a constitutive promoter. Thus cells transformed by pEZC629 are resistant to chloramphenicol, because of the chloramphenicol acetyl transferase gene on the same segment as *tetR,* but are sensitive to kanamycin. The reactions result in separation of the *tetR* gene from the regulated kanamycin resistance gene. This separation results in kanamycin resistance in cells receiving the desired recombination product. The first and the second recombination events are driven by the addition of the same recombinase, Cre.

[0040] **Figure 3B** depicts a restriction map of EZC6Bypr.

[0041] **Figure 3C** depicts a restriction map of EZC6prod.

[0042] **Figure 3D** depicts a restriction map of pEZC602.

[0043] **Figure 3E** depicts a restriction map of pEZC629.

[0044] **Figure 3F** depicts a restriction map of EZC6coint.

[0045] **Figure 4A** depicts an application of the *in vitro* method of recombinational cloning to subclone the chloramphenicol acetyl transferase gene into a vector for expression in eukaryotic cells. The Insert Donor plasmid, pEZC843, is comprised of the chloramphenicol acetyl transferase gene of *E. coli,* cloned between *loxP* and *attB* sites such that the *loxP* site is positioned at the 5'-end of the gene. The Vector Donor plasmid, pEZC1003, contains the cytomegalovirus eukaryotic promoter apposed to a *loxP* site. The supercoiled plasmids were combined with lambda Integrase and Cre recombinase *in vitro.* After incubation, competent *E. coli* cells were transformed with the recombinational reaction solution. Aliquots of transformations were spread on agar plates containing kanamycin to select for the Product molecule (here CMVProd).

[0082] Insert: is the desired DNA segment (segment ***A*** of Figure 1) which one wishes to manipulate by the method of the present invention. The insert can have one or more genes.

[0083] Insert Donor: is one of the two parental DNA molecules of the present invention which carries the Insert. The Insert Donor DNA molecule comprises the Insert flanked on both sides with recombination signals. The Insert Donor can be linear or circular. In one embodiment of the invention, the Insert Donor is a circular DNA molecule and further comprises a cloning vector sequence outside of the recombination signals (see Figure 1).

[0084] Product: is one or both the desired daughter molecules comprising the ***A*** and ***D*** or ***B*** and ***C*** sequences which are produced after the second recombination event during the recombinational cloning process (see Figure 1). The Product contains the DNA which was to be cloned or subcloned.

[0085] Promoter: is a DNA sequence generally described as the 5'-region of a gene, located proximal to the start codon. The transcription of an adjacent DNA segment is initiated at the promoter region. A repressible promoter's rate of transcription decreases in response to a repressing agent. An inducible promoter's rate of transcription increases in response to an inducing agent. A constitutive promoter's rate of transcription is not specifically regulated, though it can vary under the influence of general metabolic conditions.

[0086] Recognition sequence: Recognition sequences are particular DNA sequences which a protein, DNA, or RNA molecule (*e.g.*, restriction endonuclease, a modification methylase, or a recombinase) recognizes and binds. For example, the recognition sequence for Cre recombinase is *loxP* which is a 34 base pair sequence comprised of two 13 base pair inverted repeats (serving as the recombinase binding sites) flanking an 8 base pair core sequence. *See* Figure 1 of Sauer, B., *Current Opinion in Biotechnology* 5:521-527 (1994). Other examples of recognition sequences are the *attB, attP, dttL,* and *attR* sequences which are recognized by the recombinase enzyme λ Integrase. *attB* is an approximately 25 base pair sequence containing two 9 base pair core-type Int binding sites and a 7 base pair overlap region. *attP* is an approximately 240 base pair sequence containing core-type Int binding sites and arm-type Int binding sites as well as sites for auxiliary proteins IHF, FIS, and Xis. *See* Landy, *Current Opinion in Biotechnology* 3:699-707 (1993). Such sites are also engineered according to the present invention to enhance methods and products.

[0087] Recombinase: is an enzyme which catalyzes the exchange of DNA segments at specific recombination sites.

[0088] Recombinational Cloning: is a method described herein, whereby segments of DNA molecules are exchanged, inserted, replaced, substituted or modified, *in vitro* or *in vivo.*

[0089] Recombination proteins: include excisive or integrative proteins, enzymes, co-factors or associated proteins that are involved in recombination reactions involving one or more recombination sites. *See,* Landy (1994), *infra.*

[0090] Repression cassette: is a DNA segment that contains a repressor of a Selectable marker present in the subcloning vector.

[0091] Selectable marker: is a DNA segment that allows one to select for or against a molecule or a cell that contains it, often under particular conditions. These markers can encode an activity, such as, but not limited to, production of RNA, peptide, or protein, or can provide a binding site for RNA, peptides, proteins, inorganic and organic compounds or compositions and the like. Examples of Selectable markers include but are not limited to: (1) DNA segments that encode products which provide resistance against otherwise toxic compounds (*e.g.*, antibiotics); (2) DNA segments that encode products which are otherwise lacking in the recipient cell (*e.g.*, tRNA genes, auxotrophic markers); (3) DNA segments that encode products which suppress the activity of a gene product; (4) DNA segments that encode products which can be readily identified *(e.g.,* phenotypic markers such as β-galactosidase, green fluorescent protein (GFP), and cell surface proteins); (5) DNA segments that bind products which are otherwise detrimental to cell survival and/or function; (6) DNA segments that otherwise inhibit the activity of any of the DNA segments described in Nos. 1-5 above *(e.g.,* antisense oligonucleotides); (7) DNA segments that bind products that modify a substrate *(e.g.* restriction endonucleases); (8) DNA segments that can be used to isolate a desired molecule *(e.g.* specific protein binding sites); (9) DNA segments that encode a specific nucleotide sequence which can be otherwise non-functional *(e.g.,* for PCR amplification of subpopulations of molecules); and/or (10) DNA segments, which when absent, directly or indirectly confer sensitivity to particular compounds.

[0092] Selection scheme: is any method which allows selection, enrichment, or identification of a desired Product or Product(s) from a mixture containing the Insert Donor, Vector Donor, and/or any intermediates, (*e.g.* a Cointegrate) Byproducts. The selection schemes of one preferred embodiment have at least two components that are either linked or unlinked during recombinational cloning. One component is a Selectable marker. The other component controls the expression *in vitro* of the Selectable marker, or survival of the cell harboring the plasmid carrying the Selectable marker. Generally, this controlling element will be a repressor or inducer of the Selectable marker, but other means for controlling expression of the Selectable marker can be used. Whether a repressor or activator is used will depend on whether the marker is for a positive or negative selection, and the exact arrangement of the various DNA segments, as will be readily apparent to those skilled in the art. A preferred requirement is that the selection scheme results in selection of or enrichment for only one or more desired Products. As defined herein, to select for a DNA molecule includes (a) selecting or enriching for the presence of the desired DNA molecule, and (b) selecting or enriching against the presence of DNA molecules that are not the desired DNA molecule.

sites or *att* sites). Segment ***A*** or ***D*** can contain at least one Selection Marker, expression signals, origins of replication, or specialized functions for detecting, selecting, expressing, mapping or sequencing DNA, where ***D*** is used in this example.

[0103] Examples of desired DNA segments that can be part of Element ***A*** or ***D*** include, but are not limited to, PCR products, large DNA segments, genomic clones or fragments, cDNA clones, functional elements, *etc.,* and genes or partial genes, which encode useful nucleic acids or proteins. Moreover, the recombinational cloning of the present invention can be used to make *ex vivo* and *in vivo* gene transfer vehicles for protein expression and/or gene therapy.

[0104] In Figure 1, the scheme provides the desired Product as containing vectors ***D*** and ***A,*** as follows. The Insert Donor (containing ***A*** and ***B***) is first recombined at the square recombination sites by recombination proteins, with the Vector Donor (containing ***C*** and ***D***), to form a Co-integrate having each of A-D-C-B. Next, recombination occurs at the circle recombination sites to form Product DNA (***A*** and ***D***) and Byproduct DNA (***C*** and B). However, if desired, two or more different Co-integrates can be formed to generate two or more Products.

[0105] In one embodiment of the present *in vitro* or *in vivo* recombinational cloning method, a method for selecting at least one desired Product DNA is provided. This can be understood by consideration of the map of plasmid pEZC726 depicted in Figure 2. The two exemplary recombination sites are *attP* and *loxP.* On one segment defined by these sites is a kanamycin resistance gene whose promoter has been replaced by the *tetOP* operator/promoter from transposon *Tn*10. In the absence of tet repressor protein, *E. coli* RNA polymerase transcribes the kanamycin resistance gene from the *tetOP.* If tet repressor is present, it binds to *tetOP* and blocks transcription of the kanamycin resistance gene. The other segment of pEZC726 has the *tet* repressor gene expressed by a constitutive promoter. Thus cells transformed by pEZC726 are resistant to chloramphenicol, because of the chloramphenicol acetyl transferase gene on the same segment as *tetR,* but are sensitive to kanamycin. The recombination reactions result in separation of the *tetR* gene from the regulated kanamycin resistance gene. This separation results in kanamycin resistance in cells receiving the desired recombination Product.

[0106] Two different sets of plasmids were constructed to demonstrate the *in vitro* method. One set, for use with Cre recombinase only (cloning vector 602 and subcloning vector 629 (Figure 3)) contained *loxP* and *loxP 511* sites. A second set, for use with Cre and integrase (cloning vector 705 and subcloning vector 726 (Figure 2)) contained *loxP* and *att* sites. The efficiency of production of the desired daughter plasmid was about 60 fold higher using both enzymes than using Cre alone. Nineteen of twenty four colonies from the Cre-only reaction contained the desired product, while thirty eight of thirty eight colonies from the integrase plus Cre reaction contained the desired product plasmid.

[0107] ***Other Selection Schemes*** A variety of selection schemes can be used that are known in the art as they can suit a particular purpose for which the recombinational cloning is carried out. Depending upon individual preferences and needs, a number of different types of selection schemes can be used in the recombinational cloning method of the present invention. The skilled artisan can take advantage of the availability of the many DNA segments or methods for making them and the different methods of selection that are routinely used in the art. Such DNA segments include but are not limited to those which encodes an activity such as, but not limited to, production of RNA, peptide, or protein, or providing a binding site for such RNA, peptide, or protein. Examples of DNA molecules used in devising a selection scheme are given above, under the definition of "selection scheme"

[0108] Additional examples include but are not limited to:

(i) Generation of new primer sites for PCR *(e.g.,* juxtaposition of two DNA sequences that were not previously juxtaposed);

(ii) Inclusion of a DNA sequence acted upon by a restriction endonuclease or other DNA modifying enzyme, chemical, ribozyme, etc.;

(iii) Inclusion of a DNA sequence recognized by a DNA binding protein, RNA, DNA, chemical, etc.) (*e.g.*, for use as an affinity tag for selecting for or excluding from a population) (Davis, *Nucl. Acids Res. 24:*702-706 (1996); *J. Viral. 69:* 8027-8034 (1995));

(iv) *In vitro* selection of RNA ligands for the ribosomal L22 protein associated with Epstein-Barr virus-expressed RNA by using randomized and cDNA-derived RNA libraries;

(vi) The positioning of functional elements whose activity requires a specific orientation or juxtaposition (*e.g.*, (a) a recombination site which reacts poorly in trans, but when placed in cis, in the presence of the appropriate proteins, results in recombination that destroys certain populations of molecules; *(e.g.,* reconstitution of a promoter sequence that allows *in vitro* RNA synthesis). The RNA can be used directly, or can be reverse transcribed to obtain the desired DNA construct;

(vii) Selection of the desired product by size *(e.g.,* fractionation) or other physical property of the molecule(s); and

(viii) Inclusion of a DNA sequence required for a specific modification (*e.g.*, methylation) that allows its identification.

[0109] After formation of the Product and Byproduct in the method of the present invention, the selection step can be carried out either *in vitro* or *in vivo* depending upon the particular selection scheme which has been optionally

recombination, as reverse reactions of each other.

[0116] Each of the *att* sites contains a 15 bp core sequence; individual sequence elements of functional significance lie within, outside, and across the boundaries of this common core (Landy, A., *Ann. Rev. Biochem. 58*:913 (1989)). Efficient recombination between the various *att* sites requires that the sequence of the central common region be identical between the recombining partners, however, the exact sequence is now found to be modifiable. Consequently, derivatives of the *att* site with changes within the core are now discovered to recombine as least as efficiently as the native core sequences.

[0117] Integrase acts to recombine the *attP* site on bacteriophage lambda (about 240 bp) with the *attB* site on the *E. coli* genome (about 25 bp) (Weisberg, R.A. and Landy, A. in *Lambda II*, p. 211 (1983), Cold Spring Harbor Laboratory)), to produce the integrated lambda genome flanked by *attL* (about 100 bp) and *attR* (about 160 bp) sites. In the absence of Xis (see below), this reaction is essentially irreversible. The integration reaction mediated by integrase and IHF works *in vitro,* with simple buffer containing spermidine. Integrase can be obtained as described by Nash, H.A., *Methods of Enzymology 100*:210-216 (1983). IHF can be obtained as described by Filutowicz, M., *et al., Gene 147*: 149-150 (1994).

[0118] In the presence of the λ protein Xis (excise) integrase catalyzes the reaction of *attR* and *attL* to form *attP* and *attB, i.e.,* it promotes the reverse of the reaction described above. This reaction can also be applied in the present invention.

[0119] ***Other Recombination Systems.*** Numerous recombination systems from various organisms can also be used, based on the teaching and guidance provided herein. *See, e.g.,* Hoess *et al., Nucleic Acids Research 14*(6): 2287 (1986); Abremski *et al., J. Biol. Chem.261*(1):391 (1986); Campbell, *J. Bacteriol. 174*(23):7495 (1992); Qian *et al., J. Biol. Chem. 267(11):7794* (1992); Araki *et al., J. Mol. Biol. 225*(1):25 (1992)). Many of these belong to the integrase family of recombinases (Argos *et al. EMBO J 5*:433-440 (1986)). Perhaps the best studied of these are the Integrase/*att* system from bacteriophage λ (Landy, A. (1993) *Current Opinions in Genetics and Devel. 3*:699-707), the Cre/*loxP* system from bacteriophage P1 (Hoess and Abremski (1990) In *Nucleic Acids and Molecular Biology,* vol. 4. Eds.: Eckstein and Lilley, Berlin-Heidelberg: Springer-Verlag; pp. 90-109), and the FLP/FRT system from the Saccharomyces cerevisiae 2 μ circle plasmid (Broach *et al. Cell 29:227-234* (1982)).

[0120] Members of a second family of site-specific recombinases, the resolvase family (*e.g.*, γδ, Tn3 resolvase, Hin, Gin, and Cin) are also known. Members of this highly related family of recombinases are typically constrained to intramolecular reactions *(e.g.,* inversions and excisions) and can require host-encoded factors. Mutants have been isolated that relieve some of the requirements for host factors (Maeser and Kahnmann (1991) *Mol. Gen. Genet. 230*: 170-176), as well as some of the constraints of intramolecular recombination.

[0121] Other site-specific recombinases similar to λ Int and similar to P1 Cre can be substituted for Int and Cre. Such recombinases are known. In many cases the purification of such other recombinases has been described in the art. In cases when they are not known, cell extracts can be used or the enzymes can be partially purified using procedures described for Cre and Int.

[0122] While Cre and Int are described in detail for reasons of example, many related recombinase systems exist and their application to the described invention is also provided according to the present invention. The integrase family of site-specific recombinases can be used to provide alternative recombination proteins and recombination sites for the present invention, as site-specific recombination proteins encoded by bacteriophage lambda, phi 80, P22, P2, 186, P4 and P1. This group of proteins exhibits an unexpectedly large diversity of sequences. Despite this diversity, all of the recombinases can be aligned in their C-terminal halves.

[0123] A 40-residue region near the C terminus is particularly well conserved in all the proteins and is homologous to a region near the C terminus of the yeast 2 mu plasmid Flp protein. Three positions are perfectly conserved within this family: histidine, arginine and tyrosine are found at respective alignment positions 396, 399 and 433 within the well-conserved C-terminal region. These residues contribute to the active site of this family of recombinases, and suggest that tyrosine-433 forms a transient covalent linkage to DNA during strand cleavage and rejoining. *See, e.g.,* Argos, P. *et al., EMBO J. 5*:433-40 (1986).

[0124] Alternatively, IS231 and other Bacillus thuringiensis transposable elements could be used as recombination proteins and recombination sites. Bacillus thuringiensis is an entomopathogenic bacterium whose toxicity is due to the presence in the sporangia of delta-endotoxin crystals active against agricultural pests and vectors of human and animal diseases. Most of the genes coding for these toxin proteins are plasmid-borne and are generally structurally associated with insertion sequences (IS231, IS232, IS240, ISBT1 and ISBT2) and transposons (Tn4430 and Tn5401). Several of these mobile elements have been shown to be active and participate in the crystal gene mobility, thereby contributing to the variation of bacterial toxicity.

[0125] Structural analysis of the iso-IS231 elements indicates that they are related to IS 1151 from *Clostridium perfringens* and distantly related to IS4 and IS186 from *Escherichia coli.* Like the other IS4 family members, they contain a conserved transposase-integrase motif found in other IS families and retroviruses.

[0126] Moreover, functional data gathered from IS231A in Escherichia coli indicate a non-replicative mode of trans-

recombination, said enhancement selected from the group consisting of substantially (i) favoring excisive integration; (ii) favoring excisive recombination; (ii) relieving the requirement for host factors; (iii) increasing the efficiency of said Cointegrate DNA or Product DNA formation; and (iv) increasing the specificity of said Cointegrate DNA or Product DNA formation.

[0136] The nucleic acid molecule preferably comprises at least one recombination site derived from attB, attP, attL or attR. More preferably the att site is selected from att1, att2, or att3, as described herein.

[0137] In a preferred embodiment, the core region comprises a DNA sequence selected from the group consisting of:

(a) RKYCWGCTTTYKTRTACNAASTSGB (m-att) (SEQ ID NO:1);

(b) AGCCWGCTTTYKTRTACNAACTSGB (m-attB) (SEQ ID NO:2);

(c) GTTCAGCTTTCKTRTACNAACTSGB (m-attR) (SEQ ID NO:3);

(d) AGCCWGCTTTCKTRTACNAAGTSGB (m-attL) (SEQ ID NO:4);

(e) GTTCAGCTTTYKTRTACNAAGTSGB(m-attP1) (SEQ ID NO:5);

or a corresponding or complementary DNA or RNA sequence, wherein R=A or G; K=G or T/U; Y=C or T/U; W=A or T/U; N=A or C or G or T/U; S=Cor G; and B=C or G or T/U, as presented in 37 C.F.R. §1.822, wherein the core region does not contain a stop codon in one or more reading frames.

[0138] The core region also preferably comprises a DNA sequence selected from the group consisting of:

(a) AGCCTGCTTTTTTGTACAAACTTGT (attB1) (SEQ ID NO:6);

(b) AGCCTGCTTTCTTGTACAAACTTGT (attB2) (SEQ ID NO:7);

(c) ACCCAGCTTTCTTGTACAAACTTGT (attB3) (SEQ ID NO:8);

(d) GTTCAGCTTTTTTGTACAAACTTGT (attR1) (SEQ ID NO:9);

(e) GTTCAGCTTTCTTGTACAAACTTGT (attR2) (SEQID NO:10);

(f) GTTCAGCTTTCTTGTACAAAGTTGG (attR3) (SEQ ID NO:11);

Example 1: Recombinational Cloning Using Cre and Cre & Int

[0149] Two pairs of plasmids were constructed to do the *in vitro* recombinational cloning method in two different ways. One pair, pEZC705 and pEZC726 (Figure 2A), was constructed with *loxP* and *att* sites, to be used with Cre and λ integrase. The other pair, pEZC602 and pEZC629 (Figure 3A), contained the *loxP* (wild type) site for Cre, and a second mutant *lox* site, *loxP 511,* which differs from *loxP* in one base (out of 34 total). The minimum requirement for recombinational cloning of the present invention is two recombination sites in each plasmid, in general ***X*** and ***Y,*** and ***X'*** and ***Y'***. Recombinational cloning takes place if either or both types of site can recombine to form a Cointegrate *(e. g.* ***X*** and ***X'***), and if either or both (but necessarily a site different from the type forming the Cointegrate) can recombine to excise the Product and Byproduct plasmids from the Cointegrate *(e.g.* ***Y*** and ***Y'***). It is important that the recombination sites on the same plasmid do not recombine. It was found that the present recombinational cloning could be done with Cre alone.

Cre-Only

[0150] Two plasmids were constructed to demonstrate this conception *(see* Figure 3A). pEZC629 was the Vector Donor plasmid. It contained a constitutive drug marker (chloramphenicol resistance), an origin of replication, *loxP* and *loxP 511* sites, a conditional drug marker (kanamycin resistance whose expression is controlled by the operator/promoter of the tetracycline resistance operon of transposon *Tn*10) and a constitutively expressed gene for the tet repressor protein, *tetR. E. coli* cells containing pEZC629 were resistant to chloramphenicol at 30 μg/ml, but sensitive to kanamycin at 100 μg/ml. pEZC602 was the Insert Donor plasmid, which contained a different drug marker (ampicillin resistance), an origin, and *loxP* and *loxP 511* sites flanking a multiple cloning site.

[0151] This experiment was comprised of two parts as follows:

Part I: About 75 ng each of pEZC602 and pEZC629 were mixed in a total volume of 30 μl of Cre buffer (50 mM Tris-HCl pH 7.5, 33 mM NaCl, 5 mM spermidine-HCl, 500 μg/ml bovine serum albumin). Two 10 μl aliquots were transferred to new tubes. One tube received 0.5 μl of Cre protein (approx. 4 units per μl; partially purified according to Abremski and Hoess, *J. Biol. Chem. 259*:1509 (1984)). Both tubes were incubated at 37°C for 30 minutes, then 70°C for 10 minutes. Aliquots of each reaction were diluted and transformed into DH5α. Following expression, aliquots were plated on 30 μg/ml chloramphenicol; 100 μg/ml ampicillin plus 200 μg/ml methicillin; or 100 μg/ml kanamycin. ***Results:*** *See* Table 1. The reaction without Cre gave 1.11×10^6 ampicillin resistant colonies (from the Insert Donor plasmid pEZC602); 7.8×10^5 chloramphenicol resistant colonies (from the Vector Donor plasmid pEZC629); and 140 kanamycin resistant colonies (background). The reaction with added Cre gave 7.5×10^5 ampicillin resistant colonies (from the Insert Donor plasmid pEZC602); 6.1×10^5 chloramphenicol resistant colonies (from the Vector Donor plasmid pEZC629); and 760 kanamycin resistant colonies (mixture of background colonies and colonies from the recombinational cloning Product plasmid). ***Analysis:*** Because the number of colonies on the kanamycin plates was much higher in the presence of Cre, many or most of them were predicted to contain the desired Product plasmid.

Table 1

Enzyme	Ampicillin	Chloramphenicol	Kanamycin	Efficiency
None	1.1×10^6	7.8×10^5	140	$140/7.8 \times 10^5 = 0.02\%$
Cre	7.5×10^5	6.1×10^5	760	$760/6.1 \times 10^5 = 0.12\%$

[0152] ***Part II:*** Twenty four colonies from the "+ Cre" kanamycin plates were picked and inoculated into medium containing 100 μg/ml kanamycin. Minipreps were done, and the miniprep DNAs, uncut or cut with *Sma*I or *Hin*dIII, were electrophoresed. ***Results:*** 19 of the 24 minipreps showed supercoiled plasmid of the size predicted for the Product plasmid. All 19 showed the predicted *Sma*I and *Hin*dIII restriction fragments. ***Analysis:*** The Cre only scheme was demonstrated. Specifically, it was determined to have yielded about 70% (19 of 24) Product clones. The efficiency was about 0.1% (760 kanamycin resistant clones resulted from 6.1×10^5 chloramphenicol resistant colonies).

Cre Plus Integrase

[0153] The plasmids used to demonstrate this method are exactly analogous to those used above, except that pEZC726, the Vector Donor plasmid, contained an *attP* site in place of *loxP 511,* and pEZC705, the Insert Donor plasmid, contained an *attB* site in place of *loxP 511* (Figure 2A).

(5026 base pairs), CMVProd. Restriction digestion with *NcoI* gave the fragments predicted for the chloramphenicol acetyl transferase cloned downstream of the CMV promoter for all six plasmids.

Example 3: Subcloned DNA Segments Flanked by attB Sites Without Stop Codons

Part I: Background

[0156] The above examples are suitable for transcriptional fusions, in which transcription crosses recombination sites. However, both attR and loxP sites contain multiple stop codons on both strands, so translational fusions can be difficult, where the coding sequence must cross the recombination sites, (only one reading frame is available on each strand of loxP sites) or impossible (in attR or attL).

[0157] A principal reason for subcloning is to fuse protein domains. For example, fusion of the glutathione S-transferase (GST) domain to a protein of interest allows the fusion protein to be purified by affinity chromatography on glutathione agarose (Pharmacia, Inc., 1995 catalog). If the protein of interest is fused to runs of consecutive histidines (for example His6), the fusion protein can be purified by affinity chromatography on chelating resins containing metal ions (Qiagen, Inc.). It is often desirable to compare amino terminal and carboxy terminal fusions for activity, solubility, stability, and the like.

[0158] The attB sites of the bacteriophage λ integration system were examined as an alternative to loxP sites, because they are small (25 bp) and have some sequence flexibility (Hash, H.A. *et al., Proc. Natl. Acad. Sci. USA 84*: 4049-4053 (1987). It was not previously suggested that multiple mutations to remove all stop codes would result in useful recombination sites for recombinational subcloning.

[0159] Using standard nomenclature for site specific recombination in lambda bacteriophage (Weisber, in *Lambda III*, Hendrix, *et al.*, eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989)), the nucleotide regions that participate in the recombination reaction in an *E. coli* host cell are represented as follows:

```
attP  --P1--H1--P2--X--H2--C-O-C--H'--P'1--P'2--P'3--
                              +
attB                      --B-O-B'--
                  Int, IHF ⇕ Xis, Int, IHF

attR  --P1--H1--P2--X--H2--C-O-B'--
                              +
attL                      --B-O-C--H'--P'1--P'2--P'3--,
```

where: O represents the 15 bp core DNA sequence found in both the phage and *E. coli* genomes; B and B' represent approximately 5 bases adjacent to the core in the *E. coli* genome; and P1, H1, P2, X, H2, C, C', H', P'1, P'2, and P'3 represent known DNA sequences encoding protein binding domains in the bacteriophage λ genome.

[0160] The reaction is reversible in the presence of the protein Xis (excisionase); recombination between attL and attR precisely excise the λ genome from its integrated state, regenerating the circular λ genome containing attP and the linear *E. coli* genome containing attB.

Part II: Construction and Testing of Plasmids Containing Mutant att Sites

[0161] Mutant attL and attR sites were constructed. Importantly, Landy *et al.* (*Ann. Rev. Biochem. 58*:913 (1989)) observed that deletion of the P1 and H1 domains of attP facilitated the excision reaction and eliminated the integration reaction, thereby making the excision reaction irreversible. Therefore, as mutations were introduced in attR, the P1 and H1 domains were also deleted. attR sites in the present example lack the P1 and H1 regions and have the NdeI site removed (base 27630 changed from C to G), and contain sequences corresponding to bacteriophage λ coordinates 27619-27738 (GenBank release 92.0, bg:LAMCG, "Complete Sequence of Bacteriophage Lambda").

[0162] The sequence of attB produced by recombination of wild type attL and attR sites is:

Table 3 (continued)

Vector donor att site	Gene donor att site	# of kanamycin resistant colonies*
"	attLwt (pEZC1313)	0
"	attL2 (pEZC1317)	0
"	attL2 (pEZC1321)	209

(*1% of each transformation was spread on a kanamycin plate.)

[0166] The above data show that whereas the wild type att and att1 sites recombine to a small extent, the att1 and att2 sites do not recombine detectably with each other.

[0167] ***Part III.*** Recombination was demonstrated when the core region of both attB sites flanking the DNA segment of interest did not contain stop codons. The physical state of the participating plasmids was discovered to influence recombination efficiency.

[0168] The appropriate att sites were moved into pEZC705 and pEZC726 to make the plasmids pEZC1405 (Figure 5G) (attR1 and attR2) and pEZC1502 (Figure 5H) (attL1 and attL2). The desired DNA segment in this experiment was a copy of the chloramphenicol resistance gene cloned between the two attL sites of pEZC1502. Pairs of plasmids were recombined *in vitro* using Int, Xis, and IHF (no Cre because no loxP sites were present). The yield of desired kanamycin resistant colonies was determined when both parental plasmids were circular, or when one plasmid was circular and the other linear as presented in Table 4:

Table 4

Vector donor[1]	Gene donor[1]	Kanamycin resistant colonies[2]
Circular pEZC1405	None	30
Circular pEZC1405	Circular pEZC1502	2680
Linear pEZC1405	None	90
Linear pEZC1405	Circular pEZC1502	172000
Circular pEZC1405	Linear pEZC1502	73000

[1] DNAs were purified with Qiagen columns, concentrations determined by A260, and linearized with Xba I (pEZC1405) or AlwN I (pEZC1502). Each reaction contained 100 ng of the indicated DNA. All reactions (10 µl total) contained 3 µl of enzyme mix (Xis, Int, and IHF). After incubation (45 minutes at 25°, 10 minutes at 65°), one µl was used to transform *E. coli* DH5α cells.

[2]Number of colonies expected if the entire transformation reaction (1 ml) had been plated. Either 100 µl or 1 µl of the transformations were actually plated.

[0169] ***Analysis:*** Recombinational cloning using mutant attR and attL sites was confirmed. The desired DNA segment is subcloned between attB sites that do not contain any stop codons in either strand. The enhanced yield of Product DNA (when one parent was linear) was unexpected because of earlier observations that the excision reaction was more efficient when both participating molecules were supercoiled and proteins were limiting (Nunes-Duby *et al., Cell 50*:779-788 (1987).

Example 4: Demonstration of Recombinational Cloning Without Inverted Repeats

Part I: Rationale

[0170] The above Example 3 showed that plasmids containing inverted repeats of the appropriate recombination sites (for example, attL1 and attL2 in plasmid pEZC1502) (Figure 5H) could recombine to give the desired DNA segment flanked by attB sites without stop codons, also in inverted orientation. A concern was the *in vivo* and *in vitro* influence of the inverted repeats. For example, transcription of a desired DNA segment flanked by attB sites in inverted orientation could yield a single stranded RNA molecule that might form a hairpin structure, thereby inhibiting translation.

[0171] Inverted orientation of similar recombination sites can be avoided by placing the sites in direct repeat arrangement att sites. If parental plasmids each have a wild type attL and wild type attR site, in direct repeat the Int, Xis, and IHF proteins will simply remove the DNA segment flanked by those sites in an intramolecular reaction. However, the mutant sites described in the above Example 3 suggested that it might be possible to inhibit the intramolecular reaction while allowing the intermolecular recombination to proceed as desired.

[0179] In the general recombinational cloning scheme, in which the vector donor contains two segments C and D separated by recombination sites, selection for the desired product depends upon selection for the presence of segment D, and the absence of segment C. In the original Example segment D contained a drug resistance gene (Km) that was negatively controlled by a repressor gene found on segment C. When C was present, cells containing D were not resistant to kanamycin because the resistance gene was turned off.

[0180] The Dpn I gene is an example of a toxic gene that can replace the repressor gene of the above embodiment. If segment C expresses the Dpn I gene product, transforming plasmid CD into a dam+ host kills the cell. If segment D is transferred to a new plasmid, for example by recombinational cloning, then selecting for the drug marker will be successful because the toxic gene is no longer present.

Part II: Construction of a Vector Donor Using Dpn I as a Toxic Gene

[0181] The gene encoding Dpn I endonuclease was amplified by PCR using primers 5'CCA CCA CAA ACG CGT CCA TGG AAT TAC ACT TTA ATT TAG3' (SEQ. ID NO: 17) and 5'CCA CCA CAA GTC GAC GCA TGC CGA CAG CCT TCC AAA TGT3' (SEQ. ID NO: 18) and a plasmid containing the Dpn I gene (derived from plasmids obtained from Sanford A. Lacks, Brookhaven National Laboratory, Upton, New York; also available from American Type Culture Collection as ATCC 67494) as the template.

[0182] Additional mutations were introduced into the B and B' regions of attL and attR, respectively, by amplifying existing attL and attR domains with primers containing the desired base changes. Recombination of the mutant attL3 (made with oligo Xis115) and attR3 (made with oligo Xis112) yielded attB3 with the following sequence (differences from attB1 in bold):

```
        B          O          B'
ACCCA GCTTTCTTGTACAAA GTGGT   (SEQ. ID NO:8)
TGGGT CGAAAGAACATGTTT CACCA
```

The attL3 sequence was cloned in place of attL2 of an existing Gene Donor plasmid to give the plasmid pEZC2901 (Figure 7A). The attR3 sequence was cloned in place of attR2 in an existing Vector Donor plasmid to give plasmid pEZC2913 (Figure 7B) Dpn I gene was cloned into plasmid pEZC2913 to replace the tet repressor gene. The resulting Vector Donor plasmid was named pEZC3101 (Figure 7C). When pEZC3101 was transformed into the dam- strain SCS 110 (Stratagene), hundreds of colonies resulted. When the same plasmid was transformed into the dam+ strain DH5α, only one colony was produced, even though the DH5α cells were about 20 fold more competent than the SCS110 cells. When a related plasmid that did not contain the Dpn I gene was transformed into the same two cell lines, 28 colonies were produced from the SCSI 10 cells, while 448 colonies resulted from the DH5α cells. This is evidence that the Dpn I gene is being expressed on plasmid pEZC3101 (Figure 7C), and that it is killing the dam+ DH5α cells but not the dam- SCS110 cells.

Part III: Demonstration of Recombinational Cloning Using Dpn I Selection

[0183] A pair of plasmids was used to demonstrate recombinational cloning with selection for product dependent upon the toxic gene Dpn I. Plasmid pEZC3101 (Figure 7C) was linearized with Mlu I and reacted with circular plasmid pEZC2901 (Figure 7A). A second pair of plasmids using selection based on control of drug resistance by a repressor gene was used as a control: plasmid pEZC1802 (Figure 7D) was linearized with Xba I and reacted with circular plasmid pEZC1502 (Figure 5H). Eight microliter reactions containing the same buffer and proteins Xis, Int, and IHF as in previous examples were incubated for 45 minutes at 25°C, then 10 minutes at 75°C, and 1 µl aliquots were transformed into DH5α (i.e., dam+) competent cells, as presented in Table 6.

Table 6

Reaction #	Vector donor	Basis of selection	Gene donor	Colonies
1	pEZC3101/Mlu	Dpn I toxicity	---	3 .
2	pEZC3101/Mlu	Dpn I toxicity	Circular pEZC2901	4000
3	pEZC1802/Xba	Tet repressor	---	0

Part III: PCR of CAT and phoA Genes

[0190] Primers were obtained from Life Technologies, Inc., to amplify the chloramphenicol acetyl transferase (CAT) gene from plasmid pACYC184, and phoA, the alkaline phosphatase gene from *E. coli.* The primers had 12-base 5' extensions containing uracil bases, so that treatment of PCR products with uracil DNA glycosylase (UDG) would weaken base pairing at each end of the DNAs and allow the 3' strands to anneal with the protruding 3' ends of the rf1, 2, and 3 vectors described above. The sequences of the primers (all written 5' - 3') were: **CAT left,** UAU UUU CAG GGU ATG GAG AAA AAA ATC ACT GGA TAT ACC (SEQ. ID NO:27); **CAT right,** UCC CAC UUA UUA CGC CCC GCC CTG CCA CTC ATC (SEQ. ID NO:28); **phoA left,** UAU UUU CAG GGU ATG CCT GTT CTG GAA AAC CGG (SEQ. ID NO:29); and **phoA right,** UCC CAC UUA UUA TTT CAG CCC CAG GGC GGC TTT C (SEQ. ID NO:30). The primers were then used for PCR reactions using known method steps *(see, e.g.,* U.S. patent No. 5,334,515, entirely incorporated herein by reference), and the polymerase chain reaction amplification products obtained with these primers comprised the CAT or phoA genes with the initiating ATGs but without any transcriptional signals. In addition, the uracil-containing sequences on the amino termini encoded the cleavage site for TEV protease (Life Technologies, Inc.), and those on the carboxy terminal encoded consecutive TAA nonsense codons.

[0191] Unpurified PCR products (about 30 ng) were mixed with the gel purified, linear rf1, rf2, or rf3 cloning vectors (about 50 ng) in a 10 µl reaction containing 1X REact 4 buffer (LTI) and 1 unit UDG (LTI). After 30 minutes at 37°C, 1 µl aliquots of each reaction were transformed into competent *E. coli* DH5α cells (LTI) and plated on agar containing 50 µg/ml kanamycin. Colonies were picked and analysis of miniprep DNA showed that the CAT gene had been cloned in reading frame 1 (pEZC3601)(Figure 8C), reading frame 2 (pEZC3609)(Figure 8D) and reading frame 3 (pEZC3617) (Figure 8E), and that the phoA gene had been cloned in reading frame 1 (pEZC3606)(Figure 8F), reading frame 2 (pEZC3613)(Figure 8G) and reading frame 3 (pEZC3621)(Figure 8H).

Part IV: Subcloning of CAT or phoA from UDG Cloning Vectors into a GST Fusion Vector

[0192] Plasmids encoding fusions between GST and either CAT or phoA in all three reading frames were constructed by recombinational cloning as follows. Miniprep DNA of GST vector donor pEZC3501(Figure 8B) (derived from Pharmacia plasmid pGEX-2TK as described above) was linearized with Cla I. About 5 ng of vector donor were mixed with about 10 ng each of the appropriate circular gene donor vectors containing CAT or phoA in 8 µl reactions containing buffer and recombination proteins Int, Xis, and IHF (above). After incubation, 1 µl of each reaction was transformed into *E. coli* strain DH5α and plated on ampicillin, as presented in Table 7.

Table 7

DNA	Colonies (10% of each transformation)
Linear vector donor (pEZC3501/Cla)	0
Vector donor + CAT rf1	110
Vector donor + CAT rf2	71
Vector donor + CAT rf3	148
Vector donor + phoA rf1	121
Vector donor + phoA rf2	128
Vector donor + phoA rf3	31

Part V: Expression of Fusion Proteins

[0193] Two colonies from each transformation were picked into 2 ml of rich medium (CircleGrow, Bio101 Inc.) in 17 × 100 mm plastic tubes (Falcon 2059, Becton Dickinson) containing 100 µg/ml ampicillin and shaken vigorously for about 4 hours at 37°C, at which time the cultures were visibly turbid. One ml of each culture was transferred to a new tube containing 10 µl of 10% (w/v) IPTG to induce expression of GST. After 2 hours additional incubation, all cultures bad about the same turbidity; the A600 of one culture was 15. Cells from 0.35 ml each culture were harvested and treated with sample buffer (containing SDS and β-mercaptoethanol) and aliquots equivalent to about 0.15 A600 units of cells were applied to ε Novex 4-20% gradient polyacrylamide gel. Following electrophoresis the gel was stained with Coomassie blue.

[0194] ***Results:*** Enhanced expression of single protein bands was seen for all 12 cultures. The observed sizes of these proteins correlated well with the sizes predicted for GST being fused (through attB recombination sites without

tion protein.

14. A method according to Claim 13 wherein the first recombination protein recombines the first and third recombination sites and the second recombination protein recombines the second and fourth recombination sites.

15. A method according to Claim 14 wherein the first and second recombination proteins are used consecutively.

16. A method as claimed in Claim 1, wherein said recombination protein is Int.

17. A method as claimed in Claim 13, wherein the recombination protein comprises Int and IHF and, optionally, Xis.

18. Use of a nucleic acid comprising a first DNA segment and a second DNA segment, wherein the first or second segment is flanked by at least a first and a second engineered recombination sites that do not recombine with each other, in an *in vitro* method according to Claim 1.

19. Use according to Claim 18 wherein the first and second engineered recombination sites have been engineered so as to have one or more properties selected from:-

(a) enhanced specificity or efficiency of recombination;
(b) decreased reverse recombination;
(c) absence of translation stop codon; and
(d) absence of inverted repeats so as to avoid hairpin formation.

20. Use according to Claim 19 wherein enhanced specificity or efficiency of recombination is conferred by one or more properties selected from:-

(a) favoured excisive integration;
(b) favoured excisive recombination; and
(c) relieved requirement for host factors.

21. Use according to Claim 19 or 20 wherein the recombination sites are engineered to confer enhanced specificity or efficiency of recombination *in vitro.*

22. Use according to any of Claims 18-21, wherein said first recombination site is selected from the group consisting of an *att* site and a *lox* site.

23. Use according to Claim 22, wherein said second recombination site is selected from the group consisting of an *att* site and a *lox* site.

24. Use according to any of Claims 18-21, wherein a core region of at least one of the recombination sites comprises a DNA sequence selected from the group consisting of:

(a) RKYCWGCTTTYKTRTACNAASTSGB (m-att) (SEQ ID NO:1);

(b) AGCCWGCTTTYKTRTACNAACTSGB (m-attB) (SEQ ID NO:2);

(c) GTTCAGCTTTCKTRTACNAACTSGB (m-attR) (SEQ ID NO:3);

(d) AGCCWGCTTTCKTRTACNAAGTSGB (m-attL) (SEQ ID NO:4);

(e) GTTCAGCTTTYKTRTACNAAGTSGB (m-attP1) (SEQ ID NO:5);

and a corresponding or complementary DNA or RNA sequence, wherein R=A or G; K=G or T/U; Y=C or T/U; W=A or T/U; N=A or C or G or T/U; S=C or G; and B=C or G or T/U.

consisting of a cloning site, a restriction site, a promoter, an operon, an origin of replication, a functional DNA, an antisense RNA, a PCR fragment, a protein or a protein fragment.

32. Use according to Claim 30, wherein said first and second engineered recombination sites have been engineered to enhance recombination efficiency.

33. A kit comprising a container being compartmentalized and including in close confinement therein a first compartment containing a nucleic acid as defined in any of Claims 18-32, and at least one additional compartment containing at least one recombination protein capable of recombining a DNA segment comprising at least one of said recombination sites.

34. A kit according to Claim 33, comprising a first compartment containing a nucleic acid that is a Vector Donor DNA, a second compartment containing a nucleic acid that is an Insert Donor DNA molecule, and said additional compartment containing said at least one recombination protein.

35. An *in vitro* method for apposing an expression signal and a gene or partial gene comprising:

(a) mixing a first nucleic acid molecule comprising said expression signal and at least a first recombination site, and a second nucleic acid molecule comprising said gene or partial gene and at least a second recombination site; and

(b) incubating said mixture in the presence of at least one recombination protein under conditions sufficient to cause recombination of at least said first and second recombination sites thereby apposing said expression signal and said gene or partial gene.

36. A method according to Claim 35, wherein said at least one recombination protein is encoded by a bacteriophage selected from the group consisting of bacteriophage lambda, phi80, P22, P2, 186, P4 and P1.

37. A method according to Claim 35 wherein said at least one recombination protein is encoded by bacteriophage lambda.

38. A method according to any Claims 35-37 using a first nucleic acid as defined in any of Claims 18-32 and a second nucleic acid as defined in any of Claims 18-32.

39. A composition comprising a nucleic acid molecule as defined in any of Claims 18-32 and a first recombination protein.

40. A composition according to Claim 39 comprising nucleic acid molecules that are Vector Donor DNAs and Insert Donor DNAs, and said first recombination protein.

41. A composition according to Claim 39 or 40 further comprising a second recombination protein different from the first.

42. Use of a method according to any of Claims 1-17 for changing vectors, apposing promoters with genes, constructing genes for fusion proteins, changing copy number, changing replicons, cloning into phages or cloning PCR products, genomic DNAs or cDNAs,

Patentansprüche

1. Verfahren zur Herstellung eines Fusionsprodukt-DNA-Moleküls, umfassend Kombinieren *in vitro:*

(i) ein Insertion-Donor-DNA-Molekül, das ein gewünschtes DNA-Segment umfasst, das durch eine erste Rekombinationsstelle und eine zweite Rekombinationsstelle flankiert ist, wobei die erste und die zweite Rekombinationsstelle nicht miteinander rekombinieren;
(ii) ein Vektor-Donor-DNA-Molekül, das eine dritte Rekombinationsstelle und eine vierte Rekombinationsstelle enthält, wobei die dritte und die vierte Rekombinationsstelle nicht miteinander rekombinieren; und
(iii) wenigstens ein spezifisches Rekombinationsprotein, das fähig ist, die erste und dritte Rekombinationsstelle und die zweite und vierte Rekombinationsstelle zu rekombinieren;

puliert wurden, um eine oder mehrere Eigenschaften aufzuweisen, ausgewählt aus:

(a) verbesserter Spezifizität oder Rekombinationseffizient
(b) verringerter reverser Rekombination;
(c) Abwesenheit von Translationsstopcodons; und
(d) Abwesenheit von invertierten Wiederholungen, um Haarnadelbildung zu vermeiden.

20. Verwendung gemäß Anspruch 19, bei der die verbesserte Spezifizität oder Rekombinationseffizienz durch eine oder mehrere Eigenschaften übertragen werden, ausgewählt aus:

(a) bevorzugte exzisive Integration;
(b) bevorzugte exzisive Rekombination; und
(c) erleichterte Anforderung für Wirtsfaktoren.

21. Verwendung gemäß Anspruch 19 oder 20, bei der die Rekombinationsstellen genmanipuliert sind, um verbesserte Spezifizität oder Rekombinationseffizient *in vitro* zu übertragen.

22. Verwendung gemäß irgendeinem der Ansprüche 18-22, bei der die erste Rekombinationsstelle ausgewählt ist aus der Gruppe, bestehend aus einer *att*-Stelle und einer *lox*-Stelle.

23. Verwendung gemäß Anspruch 22, bei der die zweite Rekombinationsstelle ausgewählt ist aus der Gruppe, bestehend aus einer *att*-Stelle und einer *lox*-Stelle.

24. Verwendung gemäß irgendeinem der Ansprüche 18-21, bei der eine Kernregion von wenigstens einer der Rekombinationsstellen eine DNA-Sequenz umfasst, die ausgewählt ist aus der Gruppe, bestehend aus:

(a) RKYCWGCTTTYKTRTACNAASTSGB (m-att) (SEQ ID Nr.: 1);
(b) AGCCWGCTTTYKTRTACNAACTSGB (m-attB) (SEQ ID Nr.: 2);
(c) GTTCAGCTTTCKTRTACNAACTSGB (m-attR) (SEQ ID Nr.: 3);
(d) AGCCWGCTTTCKTRTACNAAGTSGB (m-attL) (SEQ ID Nr.: 4);
(e) GTTCAGCTTTYKTRTACNAAGTSGB (m-attP1) (SEQ ID Nr.: 5);

und einer entsprechenden oder komplementären DNA- oder RNA-Sequenz, wobei R=A oder G; K=G oder T/U; Y=C oder T/U; W=A oder T/U; N=A oder C oder G oder T/U; S=C oder G; und B=C oder G oder T/U.

25. Verwendung gemäß irgendeinem der Ansprüche 18 bis 21, bei der eine Kernregion von wenigstens einer der Rekombinationsstellen eine DNA-Sequenz umfasst, die ausgewählt ist aus der Gruppe, bestehend aus:

ist und ein gewünschtes DNA-Molekül umfasst.

31. Verwendung gemäß Anspruch 30, bei der das gewünschte DNA-Segment für wenigstens eines kodiert, welches ausgewählt ist aus der Gruppe, bestehend aus einer Klonierungsstelle, einer Restriktionsstelle, einem Promoter, einem Operon, einem Replikationsursprung, einer funktionellen DNA, einer Antisense-RNA, einem PCR-Fragment, einem Protein oder einem Proteinfragment.

32. Verwendung gemäß Anspruch 30, bei der die erste und zweite genmanipulierte Rekombinationsstelle genmanipuliert wurden, um Rekombinationseffizienz zu verbessern.

33. Kit, welcher ein Gefäß umfasst, das unterteilt ist und in dichter Begrenzung darin eine erste Kammer, die eine wie in irgendeinem der Ansprüche 18-32 definierte Nukleinsäure enthält, und wenigstens eine zusätzliche Kammer einschließt, die wenigstens ein Rekombinationsprotein enthält, das fähig ist, ein DNA-Segment zu rekombinieren, das wenigstens eine der Rekombinationsstellen umfasst.

34. Kit gemäß Anspruch 33, welcher eine erste Kammer, die eine Nukleinsäure enthält, die eine Vektor-Donor-DNA ist, und eine zweite Kammer umfasst, die eine Nukleinsäure enthält, die ein Insertion-Donor-DNA-Molekül ist, wobei die zusätzliche Kammer das wenigstens eine Rekombinationsprotein enthält.

35. *In vitro* Verfahren zum Appositionieren eines Expressionssignals und eines Gens oder partiellen Gens, das umfasst:

(a) Mischen eines ersten Nukleinsäuremoleküls, das das Expressionsignal und wenigstens eine erste Rekombinationsstelle umfasst, und eines zweiten Nukleinsäuremoleküls, das das Gen oder partielle Gen und wenigstens eine zweite Rekombinationsstelle umfasst; und
(b) Inkubieren der Mischung in der Gegenwart von wenigstens einem Rekombinationsprotein unter ausreichenden Bedingungen, um Rekombination von wenigstens der ersten und zweiten Rekombinationsstelle zu veranlassen, wobei dadurch das Expressionssignal und das Gens oder partielle Gen appositioniert werden.

36. Verfahren gemäß Anspruch 35, bei dem das wenigstens eine Rekombinationsprotein durch einen Bakteriophage kodiert wird, der ausgewählt ist aus der Gruppe, bestehend aus Bakteriophage Lambda, phi80, P22, P2, 186, P4 und P1.

37. Verfahren gemäß Anspruch 35, bei dem das wenigstens eine Rekombinationsprotein durch Bakteriophage Lambda kodiert wird.

38. Verfahren gemäß irgendeinem der Ansprüche 35-37 unter Verwendung einer ersten, wie in irgendeinem der Ansprüche 18-32 definierten Nukleinsäure und einer zweiten, wie in irgendeinem der Ansprüche 18-32 definierten Nukleinsäure.

39. Zusammensetzung, die ein wie in irgendeinem der Ansprüche 18-32 definiertes Nukleinsäuremolekül und ein erstes Rekombinationsprotein umfasst.

40. Zusammensetzung gemäß Anspruch 39, die Nukleinsäuremoleküle, die Vektor-Donor-DNA's und Insertion-Donor-DNA's sind, und das erste Rekombinationsprotein umfasst.

41. Zusammensetzung gemäß Anspruch 39 oder 40, die weiterhin ein zweites, zu dem ersten verschiedenes Rekombinationsprotein umfasst.

42. Verwendung eines Verfahrens gemäß irgendeinem der Ansprüche 1-17 zum Ändern von Vektoren, Appositionieren von Promotoren mit Genen, Konstruieren von Genen für Fusionsproteine, Ändern der Kopienanzahl, Ändern von Replikons, Klonieren in Phagen oder Klonieren von PCR-Produkten, Genom-DNA's oder cDNA's.

Revendications

1. Procédé de fabrication d'une molécule d'ADN formant Cointégrat, comprenant la combinaison *in vitro*

16. Procédé selon la revendication 1, dans lequel ladite protéine de recombinaison est Int.

17. Procédé selon la revendication 13, dans lequel la protéine de recombinaison comprend Int et IHF et, facultativement, Xis.

18. Utilisation d'un acide nucléique comprenant un premier segment d'ADN et un second segment d'ADN, dans laquelle soit le premier ou le second segment, est flanqué d'au moins un premier et d'un deuxième sites de recombinaison modifiés qui ne se recombinent pas l'un avec l'autre, dans un procédé *in vitro* selon la revendication 1.

19. Utilisation selon la revendication 18, dans laquelle les premier et deuxième sites de recombinaison modifiés ont été modifiés de façon à présenter une ou plusieurs des propriétés suivantes :

    (a) meilleure spécificité ou efficacité de recombinaison ;
    (b) recombinaison inverse réduite ;
    (c) absence de codon d'arrêt de traduction ; et
    (d) absence de segments à répétitions inversées, de façon à éviter la formation d'épingles à cheveux.

20. Utilisation selon la revendication 19, dans laquelle soit une meilleure spécificité ou efficacité de recombinaison est conférée par une ou plusieurs des propriétés suivantes :

    (a) intégration excisive favorisée ;
    (b) recombinaison excisive favorisée ; et
    (c) exigence réduite pour les facteurs de l'hôte.

21. Utilisation selon la revendication 19 ou 20, dans laquelle les sites de recombinaison sont modifiés pour conférer une meilleure spécificité ou efficacité de recombinaison *in vitro*.

22. Utilisation selon l'une quelconque des revendications 18 à 21, dans laquelle ledit premier site de recombinaison est choisi dans le groupe constitué d'un site *att* et d'un site *lox*.

23. Utilisation selon la revendication 22, dans laquelle ledit deuxième site de recombinaison est choisi dans le groupe constitué d'un site *att* et d'un site *lox*.

24. Utilisation selon l'une quelconque des revendications 18 à 21, dans laquelle une région centrale d'au moins l'un des sites de recombinaison comprend une séquence d'ADN choisie dans le groupe constitué par :

    (a) RKYCWGCTTTYKTRTACNAASTSGB (m-att) (SEQ ID N°:1) ;
    (b) AGCCWGCTTTYKTRTACNAACTSGB (m-attB) (SEQ ID N°:2) ;
    (c) GTTCAGCTTTCKTRTACNAACTSGB (m-attR) (SEQ ID N°:3) ;
    (d) AGCCWGCTTTCKTRTACNAAGTSGB (m-attL) (SEQ ID N°:4) ;
    (e) GTTCAGCTTTYKTRTACNAAGTSGB (m-attP1) (SEQ ID N°:5) ;

    et une séquence d'ADN ou d'ARN correspondante ou complémentaire, dans laquelle R = A ou G ; K = G ou T/U ; Y = C ou T/U ; W = A ou T/U ; N = A ou C ou G ou T/U ; S = C ou G ; et B = C ou G ou T/U.

25. Utilisation selon l'une quelconque des revendications 18 à 21, dans laquelle une région centrale d'au moins l'un des sites de recombinaison comprend une séquence d'ADN choisie dans le groupe constitué par :

un ADN fonctionnel, un ARN antisens, un fragment d'ACP, une protéine ou un fragment de protéine.

32. Utilisation selon la revendication 30, dans laquelle lesdits premier et deuxième sites de recombinaison modifiés ont été modifiés pour améliorer l'efficacité de recombinaison.

33. Kit comprenant un récipient compartimenté et renfermant dans un confinement étanche un premier compartiment contenant un acide nucléique tel que défini dans l'une quelconque des revendications 18 à 32, et au moins un compartiment supplémentaire contenant au moins une protéine de recombinaison capable de recombiner un segment d'ADN comprenant au moins l'un desdits sites de recombinaison.

34. Kit selon la revendication 33, comprenant un premier compartiment contenant un acide nucléique qui est un ADN Donneur de Vecteur, un second compartiment contenant un acide nucléique qui est une molécule d'ADN de Donneur d/Insert, et ledit compartiment supplémentaire contenant ladite protéine de recombinaison.

35. Procédé in vitro pour apposer un signal d'expression et un gène ou un gène partiel comprenant :

(a) le mélange d'une première molécule d'acide nucléique comprenant ledit signal d'expression et au moins un premier site de recombinaison et d'une seconde molécule d'acide nucléique comprenant ledit gène ou gène partiel et au moins un deuxième site de recombinaison ; et
(b) l'incubation dudit mélange en présence d'au moins une protéine de recombinaison, dans des conditions suffisantes pour provoquer la recombinaison d'au moins lesdits premier et deuxième sites de recombinaison, en apposant ainsi ledit signal d'expression et ledit gène ou gène partiel.

36. Procédé selon la revendication 35, dans lequel ladite protéine de recombinaison est codée par un bactériophage choisi dans le groupe constitué par le bactériophage lambda, le phi80, le P22, le P2, le 186, le P4 et le P1.

37. Procédé selon la revendication 35, dans lequel ladite protéine de recombinaison est codée par le bactériophage lambda.

38. Procédé selon l'une quelconque des revendications 35 à 37, utilisant un premier acide nucléique tel que défini dans l'une quelconque des revendications 18 à 32 et un second acide nucléique tel que défini dans l'une quelconque des revendications 18 à 32.

39. W Composition comprenant une molécule d'acide nucléique telle que définie dans l'une quelconque des revendications 18 à 32 et une première protéine de recombinaison.

40. Composition selon la revendication 39 comprenant des molécules d'acide nucléique qui sont des ADN de Donneur de Vecteur et des ADN de Donneur d'Insert, et ladite première protéine de recombinaison.

41. Composition selon la revendication 39 ou 40 comprenant en outre une deuxième protéine de recombinaison, différente de la première.

42. Utilisation d'un procédé selon l'une quelconque des revendications 1 à 17 pour changer des vecteurs, apposer des promoteurs à des gènes, construire des gènes pour des protéines de fusion, changer le nombre de copies, changer les réplicons, cloner en phages ou cloner des produits d'ACP, des ADN génomiques ou des ADNc.

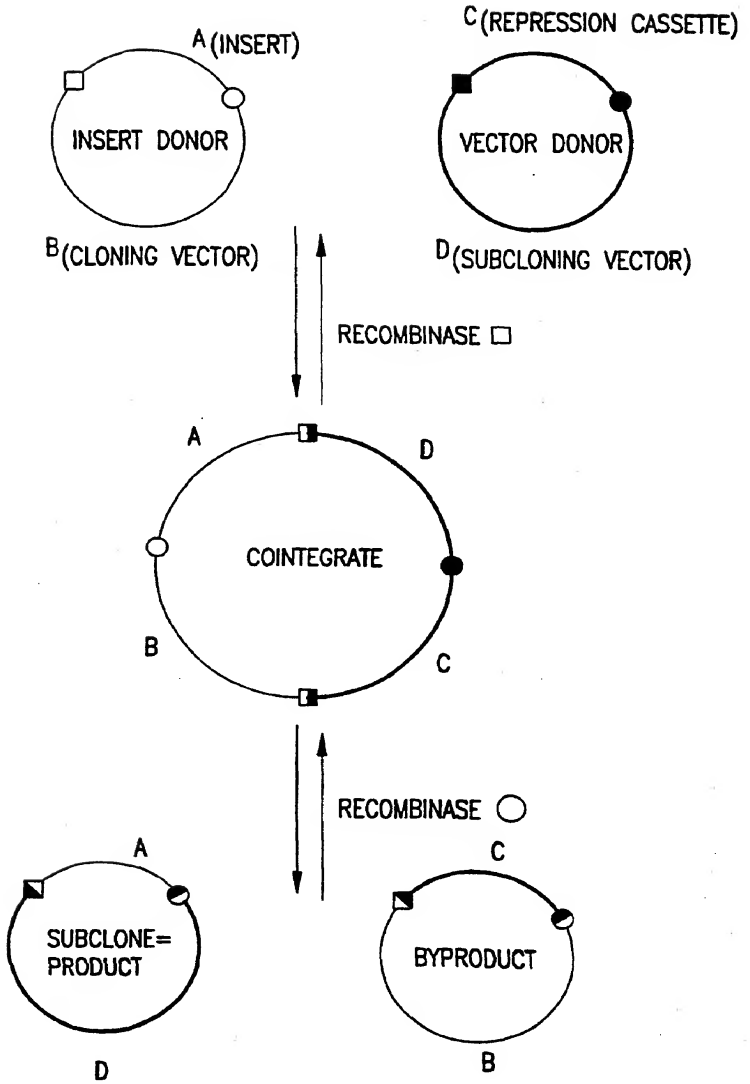

A (INSERT)
INSERT DONOR
B (CLONING VECTOR)
C (REPRESSION CASSETTE)
VECTOR DONOR
D (SUBCLONING VECTOR)
RECOMBINASE □
A
D
COINTEGRATE
B
C
RECOMBINASE ○
A
SUBCLONE=
PRODUCT
D
C
BYPRODUCT
B

FIG.1

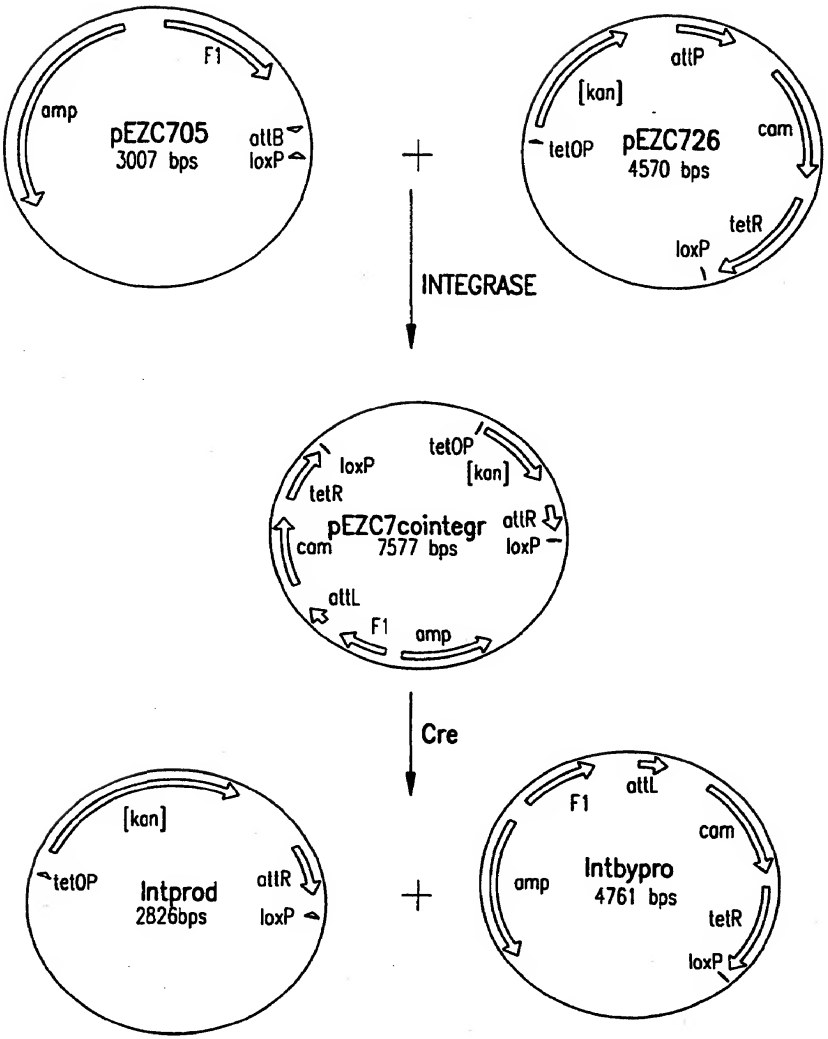

F1
amp
pEZC705
3007 bps
attB
loxP
attP
[kan]
tetOP
pEZC726
4570 bps
cam
tetR
loxP
INTEGRASE
tetOP
loxP
[kan]
tetR
pEZC7cointegr
7577 bps
attR
cam
loxP
attL
F1
amp
Cre
[kan]
tetOP
Intprod
2826bps
attR
loxP
attL
F1
cam
amp
Intbypro
4761 bps
tetR
loxP

FIG.2A

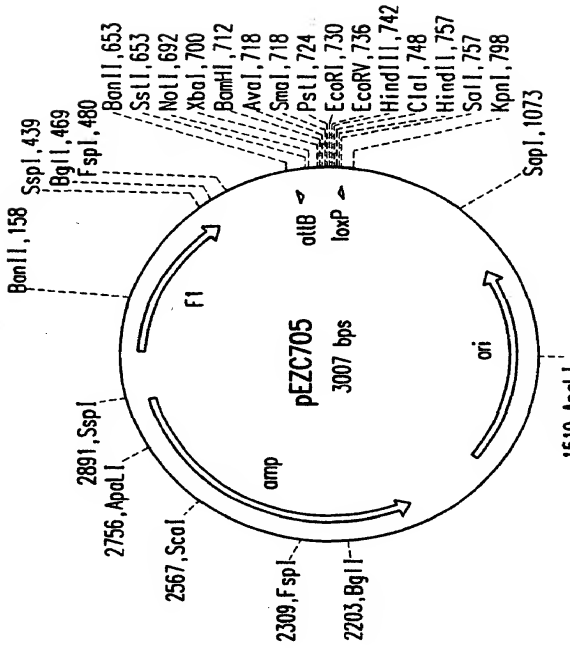

BamH1,653
Sst1,653
Not1,692
Xba1,700
BamH1,712
Ava1,718
Sma1,718
Pst1,724
EcoR1,730
EcoRV,736
Hind111,742
Cla1,748
Hind1,757
Sal1,757
Kpn1,798
Sap1,1073
1510,ApaL1
2203,Bgl1
2309,Fsp1
2567,Sca1
2756,ApaL1
2891,Ssp1
Ban11,158
Ssp1,439
Bgl1,469
Fsp1,480
F1
attB
loxP
pEZC705
3007 bps
ori
amp

FIG.2B

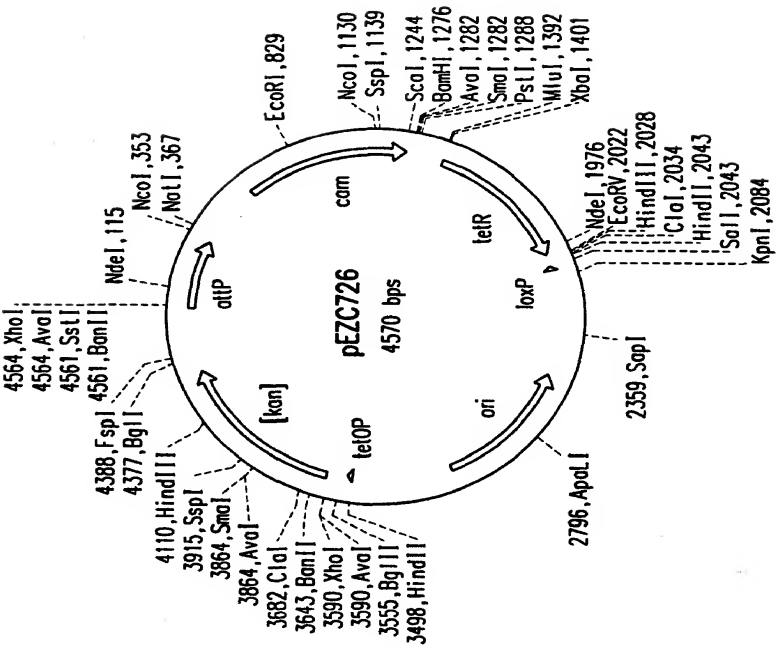

pEZC726
4570 bps
cam
tetR
loxP
ori
tetOP
[kan]
oriP
EcoRI,829
NcoI,1130
SspI,1139
ScaI,1244
BamHI,1276
AvaI,1282
SmaI,1282
PstI,1288
MluI,1392
XbaI,1401
NdeI,1976
EcoRV,2022
HindIII,2028
ClaI,2034
HindII,2043
SalI,2043
KpnI,2084
2359,SapI
2796,ApaLI
3498,HindII
3555,BglII
3590,AvaI
3590,XhoI
3643,BanII
3682,ClaI
3864,AvaI
3864,SmaI
3915,SspI
4110,HindIII
4377,BglI
4388,FspI
4561,BanII
4561,SstI
4564,AvaI
4564,XhoI
NdeI,115
NotI,367
NcoI,353

FIG.2C

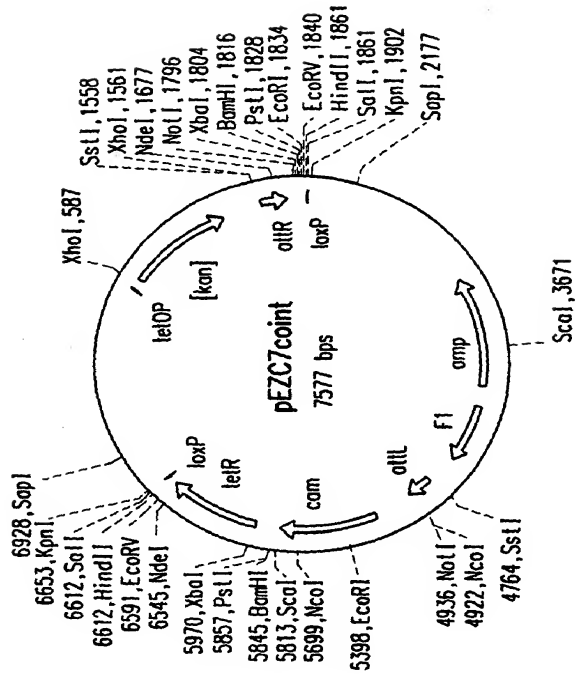

pEZC7coint
7577 bps
tetOP
[kan]
altIR
loxP
f1
omp
altL
cam
tetR
loxP
SstI,1558
XhoI,1561
NdeI,1677
NotI,1796
XbaI,1804
BamHI,1816
PstI,1828
EcoRI,1834
EcoRV,1840
HindII,1861
SalI,1861
KpnI,1902
SopI,2177
ScaI,3671
4764,SstI
4922,NcoI
4936,NotI
5398,EcoRI
5699,NcoI
5813,ScaI
5845,BamHI
5857,PstI
5970,XbaI
6545,NdeI
6591,EcoRV
6612,HindII
6612,SalI
6653,KpnI
6928,SopI
XhoI,587

FIG.2D

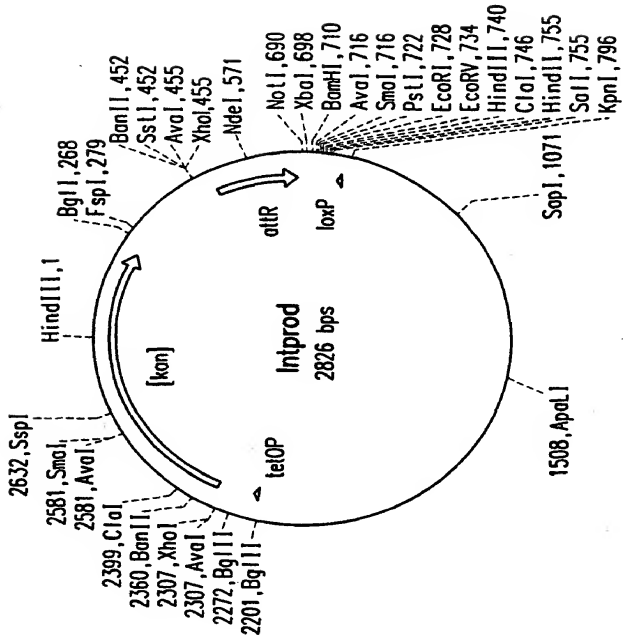

HindIII,1
Bgl I,268
Fsp I,279
BanII,452
SstI,452
AvaI,455
XhoI,455
NdeI,571
NotI,690
XbaI,698
BamHI,710
AvaI,716
SmaI,716
PstI,722
EcoRI,728
EcoRV,734
HindIII,740
ClaI,746
HindI,755
SalI,755
KpnI,796
SapI,1071
1508,ApaLI
2632,SspI
2581,SmaI,
2581,AvaI
2399,ClaI
2360,BanII,
2307,XhoI
2307,AvaI
2272,BglII
2201,BglII
tetOP
[kan]
Intprod
2826 bps
attR
loxP

FIG.2E

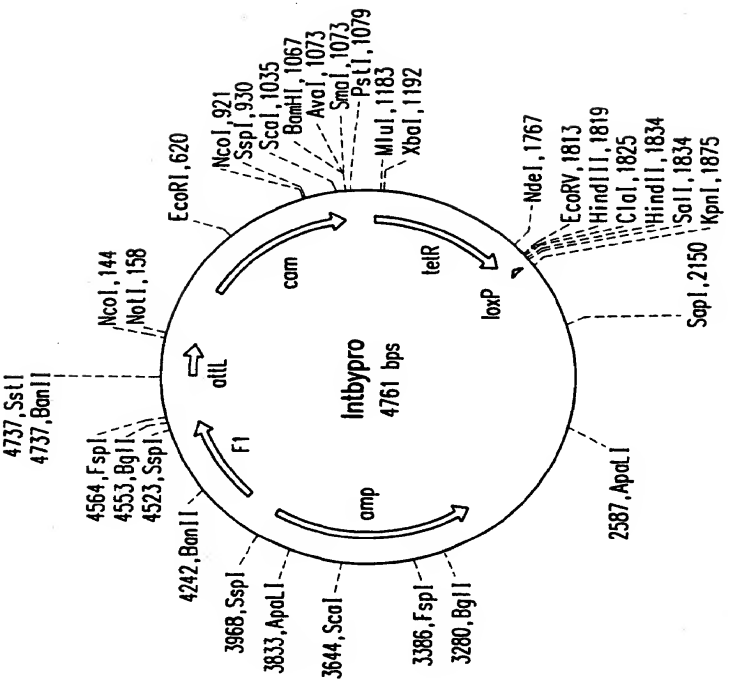

Intbypro
4761 bps
cam
tetR
loxP
amp
F1
allL
EcoRI, 620
NcoI, 921
SspI, 930
ScaI, 1035
BamHI, 1067
AvaI, 1073
SmaI, 1073
PstI, 1079
MluI, 1183
XbaI, 1192
NdeI, 1767
EcoRV, 1813
HindIII, 1819
ClaI, 1825
HindII, 1834
SalI, 1834
KpnI, 1875
SapI, 2150
2587, ApaLI
3386, FspI
3280, BglI
3644, ScaI
3833, ApaLI
3968, SspI
4242, BanII
4564, FspI
4553, BglII
4523, SspI
4737, SstI
4737, BanII
NcoI, 144
NotI, 158

FIG.2F

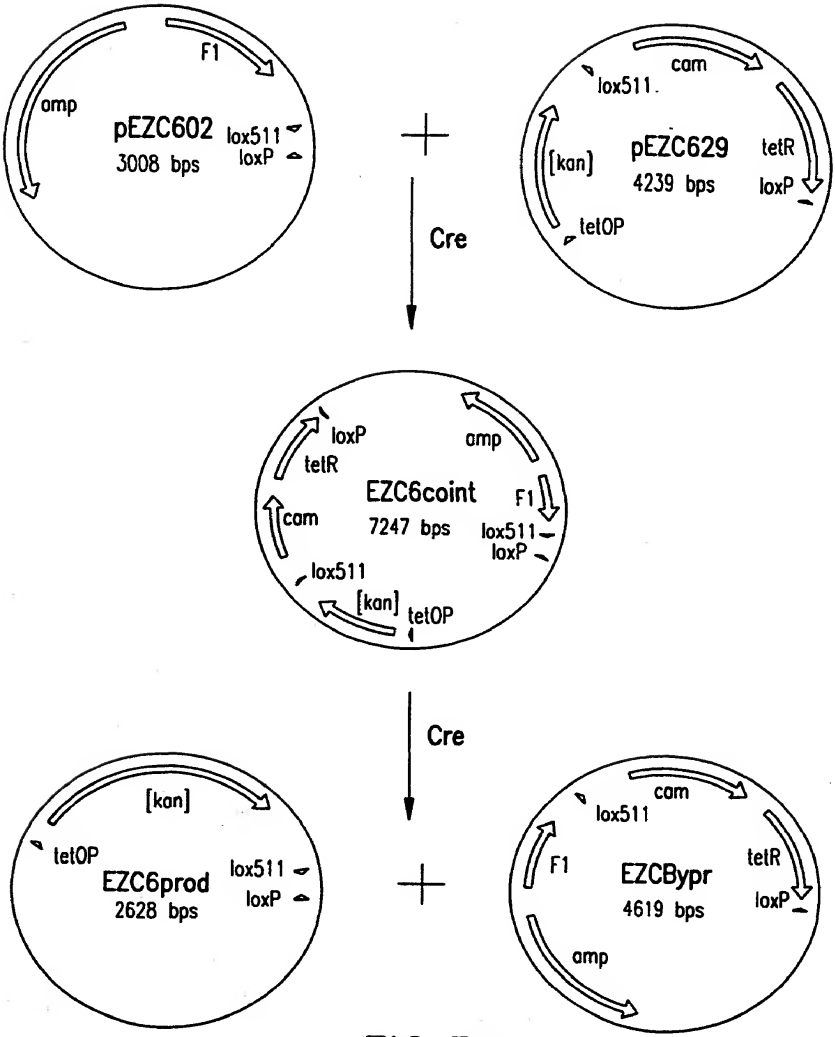

F1
amp
pEZC602
3008 bps
lox511
loxP
Cre
cam
lox511
[kan]
pEZC629
4239 bps
tetR
loxP
tetOP
loxP
amp
tetR
EZC6coint
7247 bps
F1
cam
lox511
loxP
lox511
[kan]
tetOP
Cre
[kan]
tetOP
EZC6prod
2628 bps
lox511
loxP
cam
lox511
F1
EZCBypr
4619 bps
tetR
loxP
amp

FIG.3A

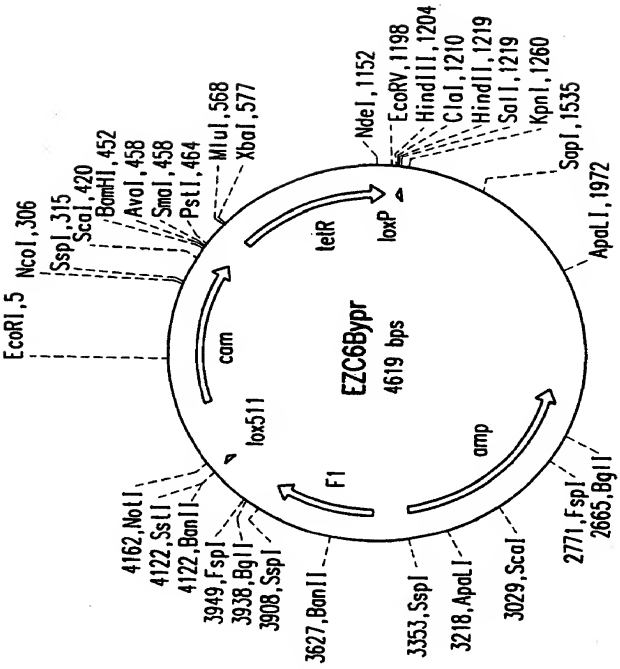

EZC66bypr
4619 bps
cam
tetR
loxP
lox511
F1
amp
EcoRI,5
NcoI,306
SspI,315
ScaI,420
BamHI,452
AvaI,458
SmaI,458
PstI,464
MluI,568
XbaI,577
NdeI,1152
EcoRV,1198
HindIII,1204
ClaI,1210
HindII,1219
SalI,1219
KpnI,1260
SapI,1535
ApaLI,1972
2665,BglI
2771,FspI
3029,ScaI
3218,ApaLI
3353,SspI
3627,BanII
3908,SspI
3938,BglI
3949,FspI
4122,BanII
4122,SstI
4162,NotI

FIG.3B

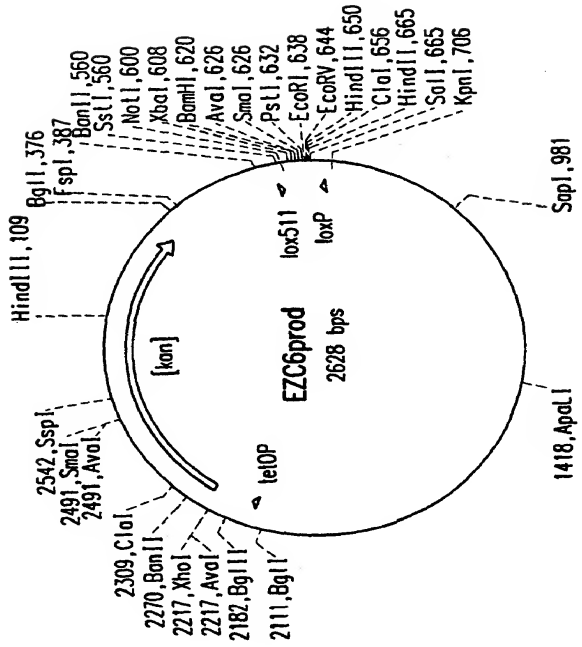
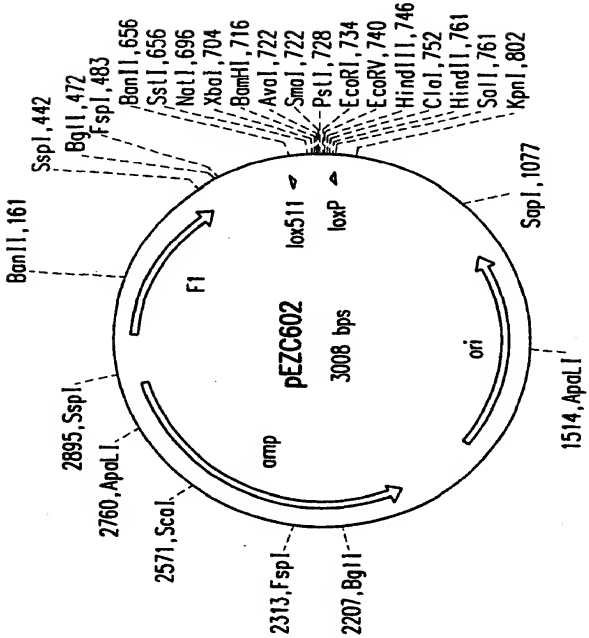

EZC6prod
2628 bps
[kan]
lox511
loxP
tetOP
HindIII,109
BglI,376
FspI,387
BanII,560
SstI,560
NotI,600
XbaI,608
BamHI,620
AvaI,626
SmaI,626
PstI,632
EcoRI,638
EcoRV,644
HindIII,650
ClaI,656
HindII,665
SalI,665
KpnI,706
SapI,981
1418,ApaLI
2111,BglI
2182,BglII
2217,AvaI
2217,XhoI
2270,BanII
2309,ClaI
2491,AvaI
2491,SmaI
2542,SspI

FIG.3C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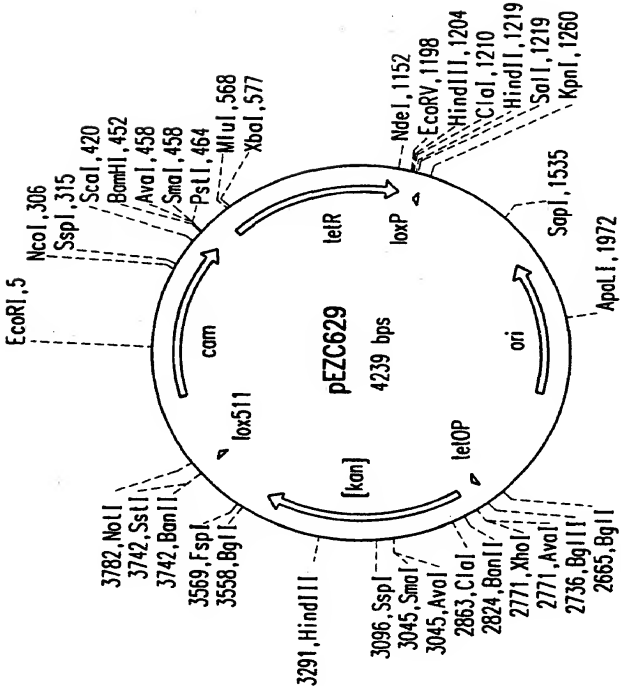
3008 bps
amp
F1
ori
lox511
loxP
BanII,161
SspI,442
BglI,472
FspI,483
BanII,656
SstI,656
NotI,696
XbaI,704
BamHI,716
AvaI,722
SmaI,722
PstI,728
EcoRI,734
EcoRV,740
HindIII,746
ClaI,752
HindII,761
SalI,761
KpnI,802
SapI,1077
1514,ApaLI
2895,SspI
2760,ApaLI
2571,ScaI
2313,FspI
2207,BglI

FIG.3D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cam
lox511
[kan]
tetOP
tetR
loxP
ori
EcoRI,5
NcoI,306
SspI,315
ScaI,420
BamHI,452
AvaI,458
SmaI,458
PstI,464
MluI,568
XbaI,577
NdeI,1152
EcoRV,1198
HindIII,1204
ClaI,1210
HindII,1219
SalI,1219
KpnI,1260
SapI,1535
ApaLI,1972
3782,NotI
3742,SstI
3742,BamII
3569,FspI
3558,BglI
3291,HindIII
3096,SspI
3045,SmaI
3045,AvaI
2863,ClaI
2824,BanII
2771,XhoI
2771,AvaII
2736,BglII
2665,BglI

FIG.3E

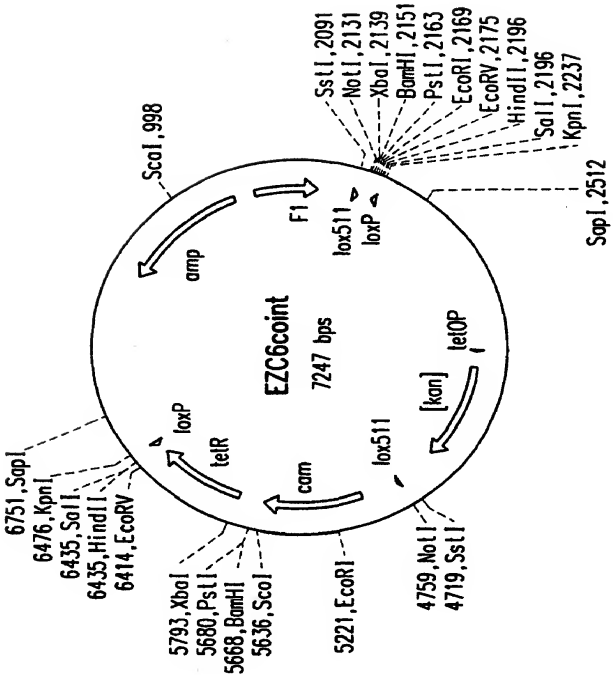
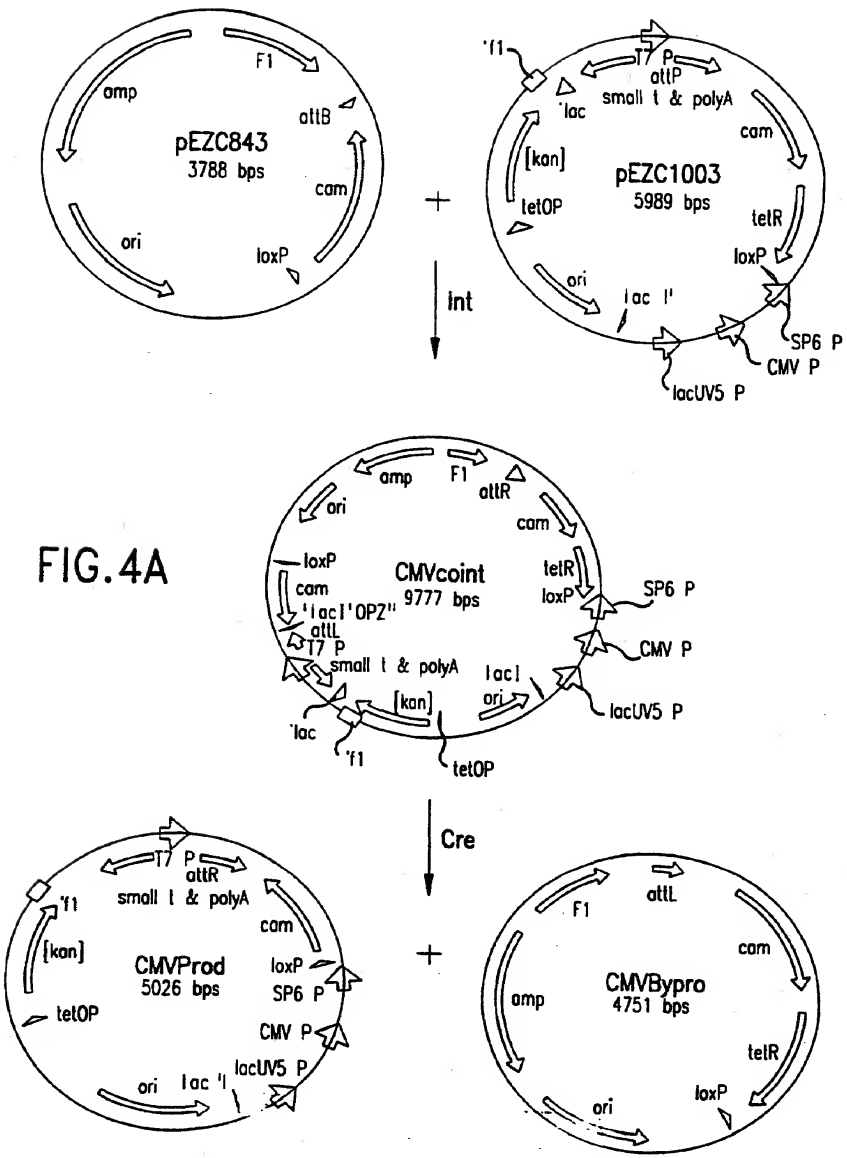

EZC6coint
7247 bps
amp
F1
lox511
loxP
tetOP
[kan]
lox511
cam
tetR
loxP
ScaI,998
SstI,2091
NotI,2131
XbaI,2139
BamHI,2151
PstI,2163
EcoRI,2169
EcoRV,2175
HindII,2196
SalI,2196
KpnI,2237
SapI,2512
4759,NotI
4719,SstI
5221,EcoRI
5636,ScaI
5668,BamHI
5680,PstI
5793,XbaI
6414,EcoRV
6435,HindII
6435,SalI
6476,KpnI
6751,SapI

FIG.3F

FIG.4A


pEZC843
3788 bps
F1
amp
attB
cam
ori
loxP
+
int
pEZC1003
5989 bps
'f1
T7 P
attP
small t & polyA
cam
'lac
[kan]
tetOP
tetR
loxP
ori
lac I'
SP6 P
CMV P
lacUV5 P
CMVcoint
9777 bps
amp
F1
attR
ori
cam
loxP
tetR
cam
'lacI'OPZ''
loxP
SP6 P
attL
T7 P
small t & polyA
CMV P
lacI
[kan]
ori
'lac
lacUV5 P
'f1
tetOP
Cre
CMVProd
5026 bps
T7 P
attR
small t & polyA
'f1
cam
[kan]
loxP
SP6 P
tetOP
CMV P
lacUV5 P
ori
lac 'I
+
CMVBypro
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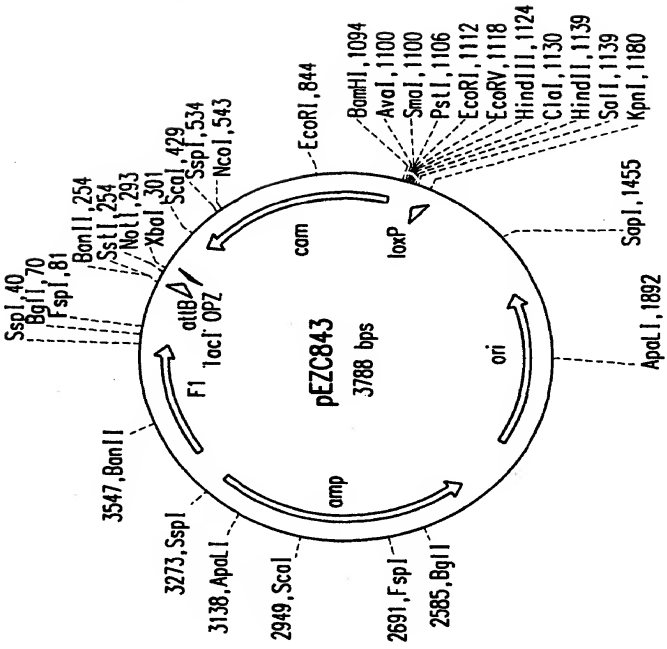
attL
F1
cam
amp
tetR
loxP
ori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cam
loxP
ori
amp
F1
lacI' OPZ
attB
EcoRI,844
BamHI,1094
AvaI,1100
SmaI,1100
PstI,1106
EcoRI,1112
EcoRV,1118
HindIII,1124
ClaI,1130
HindII,1139
SalI,1139
KpnI,1180
SapI,1455
ApaLI,1892
SspI,40
BglI,70
FspI,81
BanII,254
SstI,254
NotI,293
XbaI,301
ScaI,429
SspI,534
NcoI,543
3547,BanII
3273,SspI
3138,ApaLI
2949,ScaI
2691,FspI
2585,BglI

FIG.4B

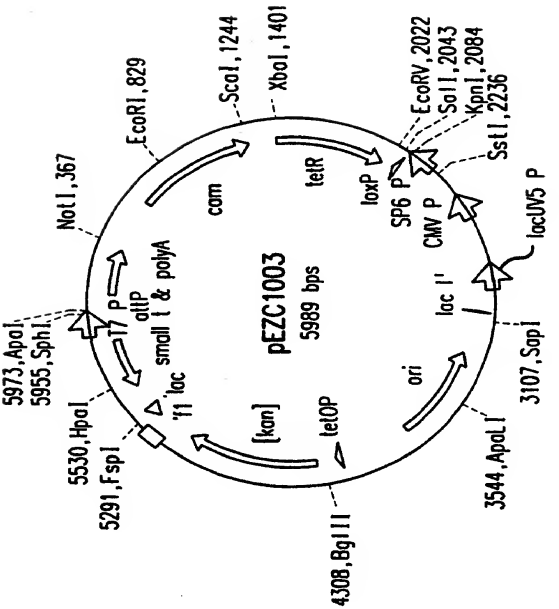
pEZC1003
5989 bps
NotI,367
EcoRI,829
ScaI,1244
XbaI,1401
EcoRV,2022
SalI,2043
KpnI,2084
SstI,2236
lacUV5 P
3107,SapI
3544,ApaLI
4308,BglII
5291,FspI
5530,HpaI
5955,SphI
5973,ApaI
T7 P
allP
small t & polyA
'I1 lac
[kan]
tetOP
ori
lac I'
CMV P
SP6 P
loxP
tetR
com

FIG.4C

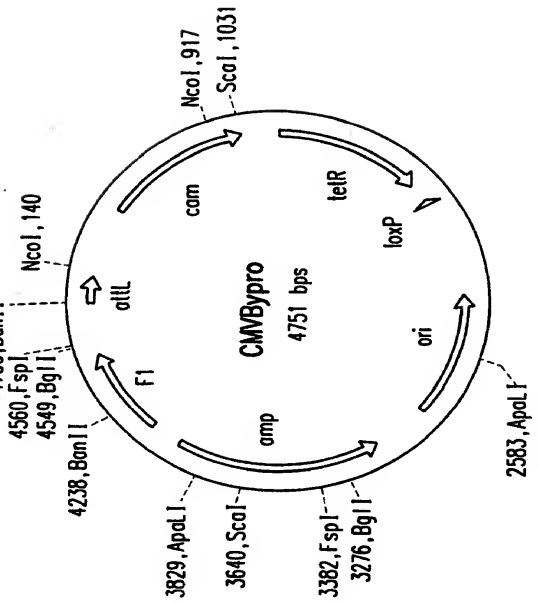
NcoI, 917
ScaI, 1031
tetR
loxP
ori
2583, ApaL I
3382, FspI
3276, BglI
3829, ApaL I
3640, ScaI
4238, BanII
4733, BanII
4560, FspI
4549, BglI
NcoI, 140
F1
ampR
CMVbpro
4751 bps
comR

FIG.4D

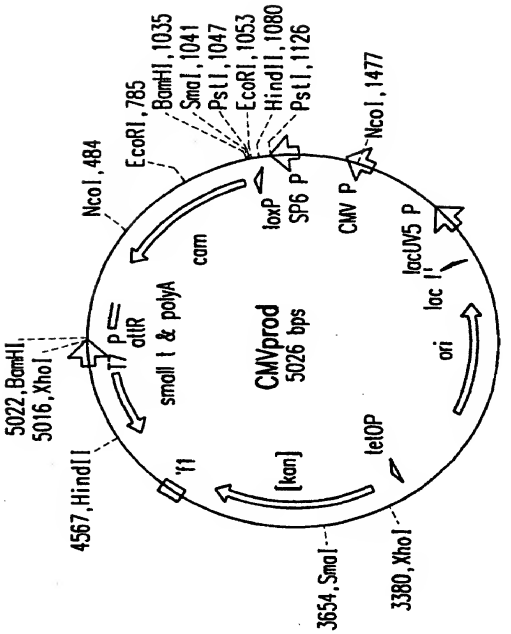
CMVprod
5026 bps
NcoI, 484
EcoRI, 785
BamHI, 1035
SmaI, 1041
PstI, 1047
EcoRI, 1053
HindII, 1080
PstI, 1126
NcoI, 1477
loxP
SP6 P
CMV P
lacUV5 P
lac I
ori
cam
smaII I & polyA
T7 P
atIR
5022, BamHI
5016, XhoI
4567, HindII
f1
[kan]
tetOP
3654, SmaI
3380, XhoI

FIG.4E

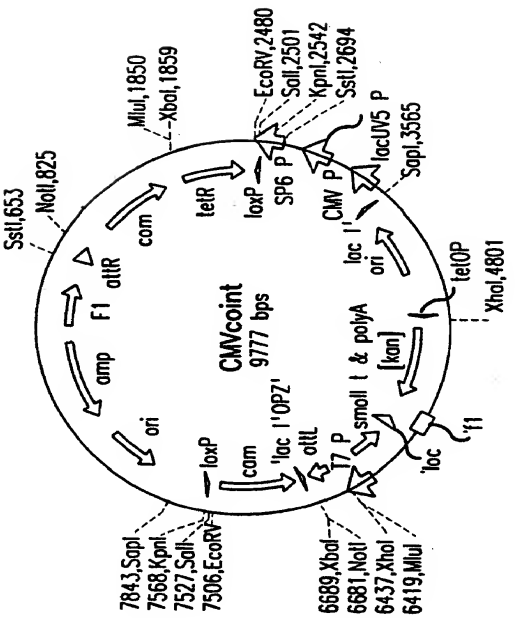
CMVcoint
9777 bps
SstII,653
NotI,825
MluI,1850
XbaI,1859
EcoRV,2480
SalI,2501
KpnI,2542
SstII,2694
SapI,3565
XhoI,4801
7843,SapI
7568,KpnI
7527,SalI
7506,EcoRV
6689,XbaI
6681,NotI
6437,XhoI
6419,MluI
amp
F1 ori
altR
com
tetR
loxP
SP6 P
CMV P
lacUV5 P
lac I'
ori
tetOP
'I1
'lac
[kan]
smallt & polyA
T7 P
altL
'lac I'OPZ'
cam
loxP
ori

FIG.4F

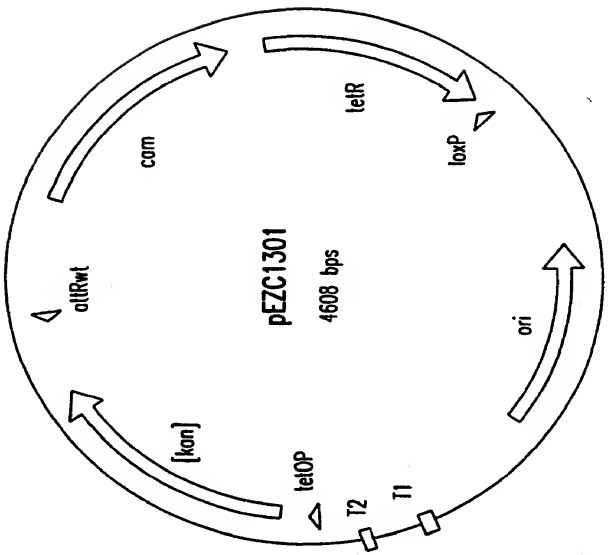

FIG.5A

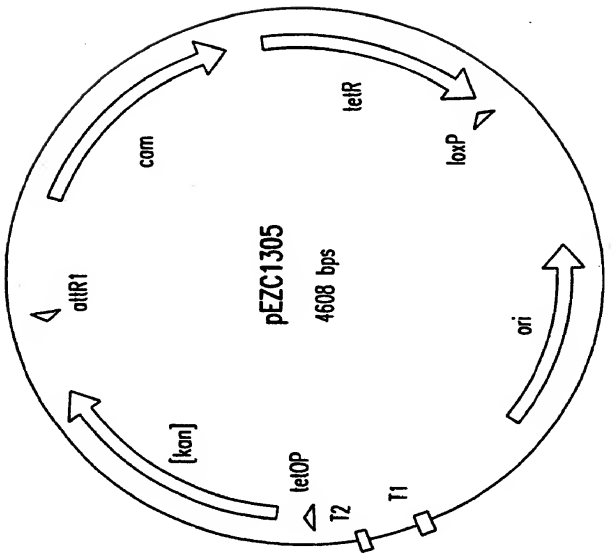

FIG.5B

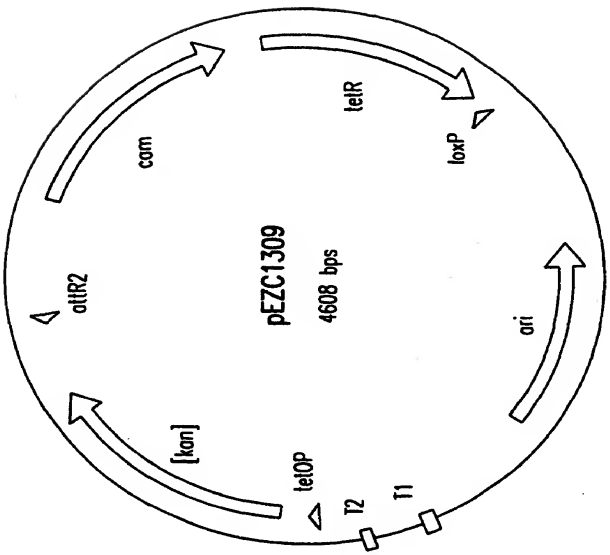

FIG.5C

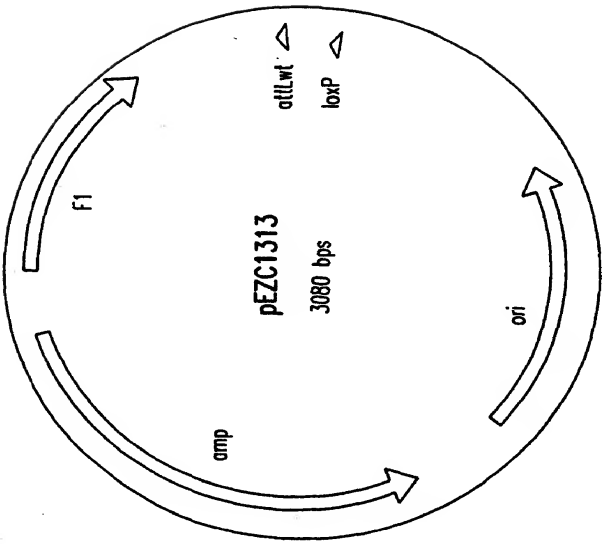

FIG.5D

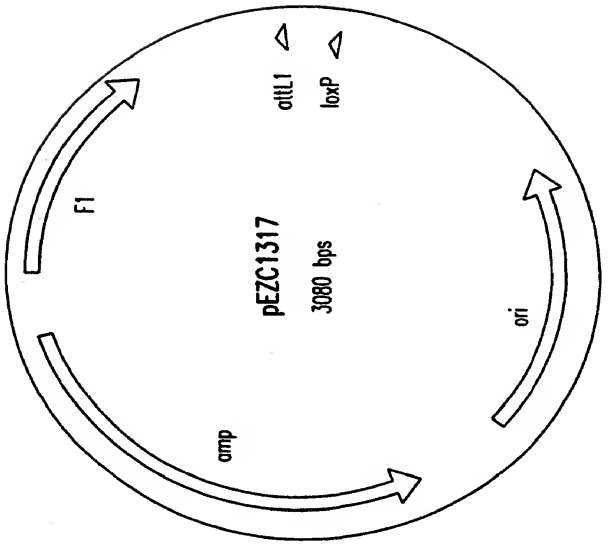

FIG.5E

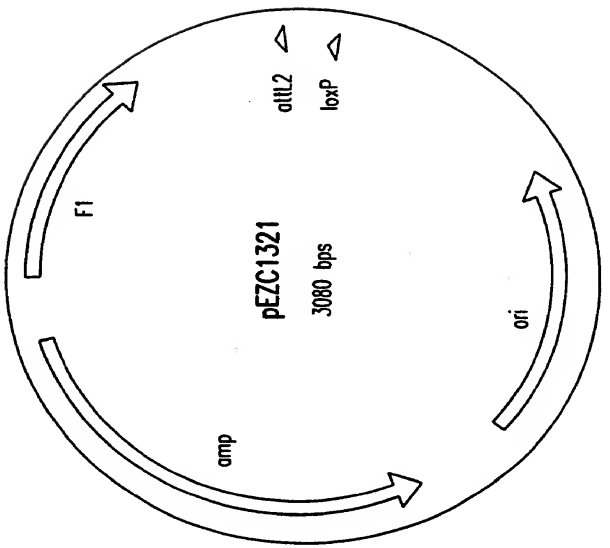

FIG.5F

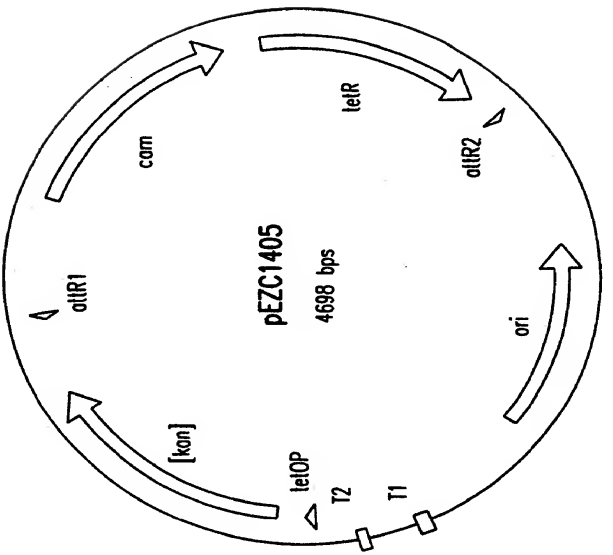

FIG.5G

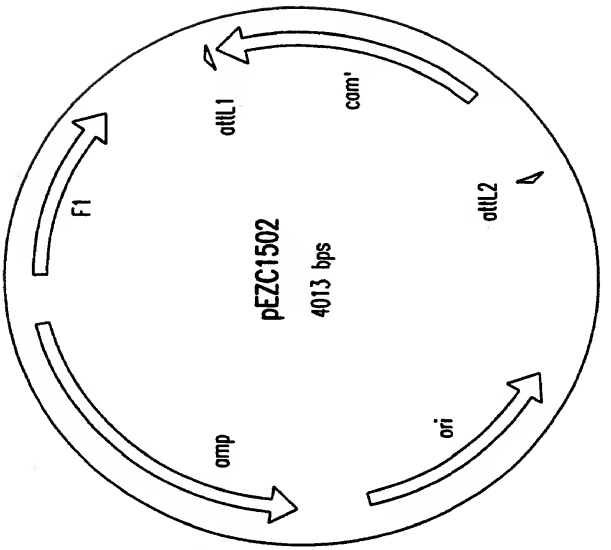

FIG.5H

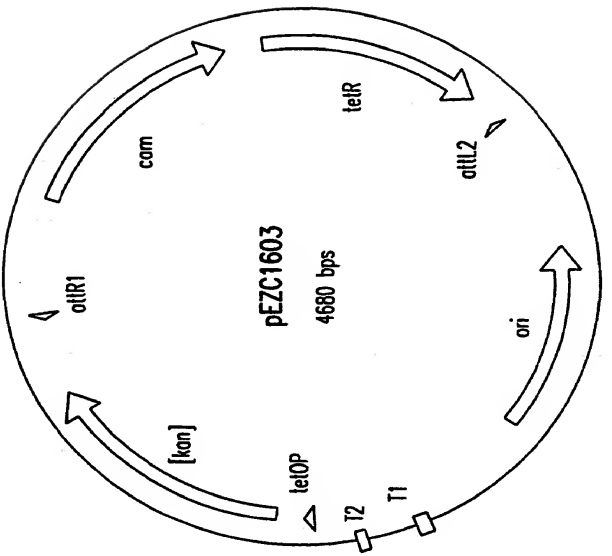

FIG.6A

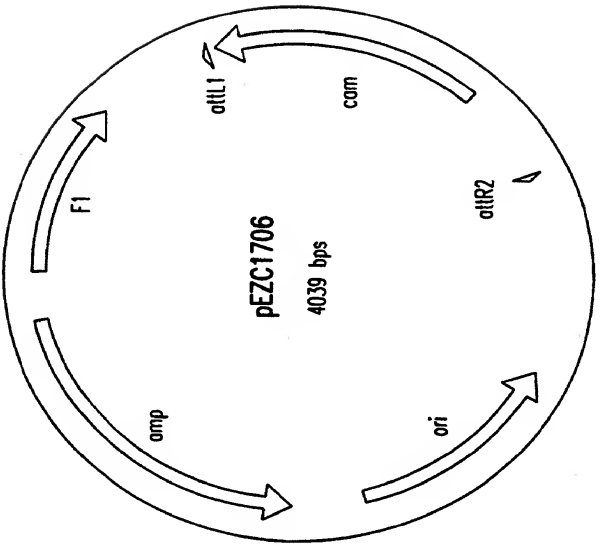

FIG.6B

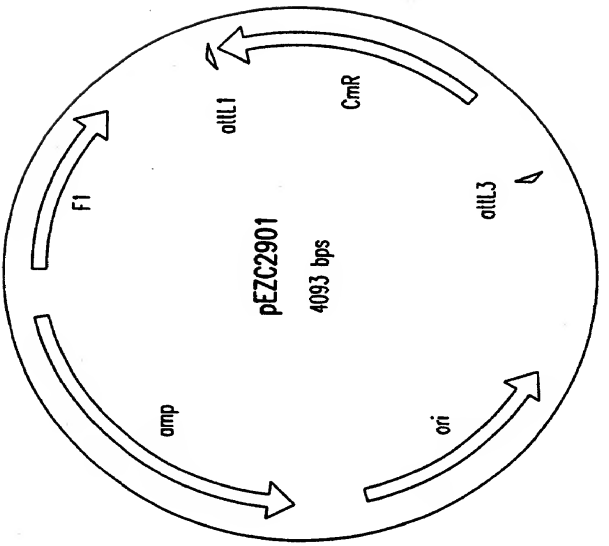

FIG.7A

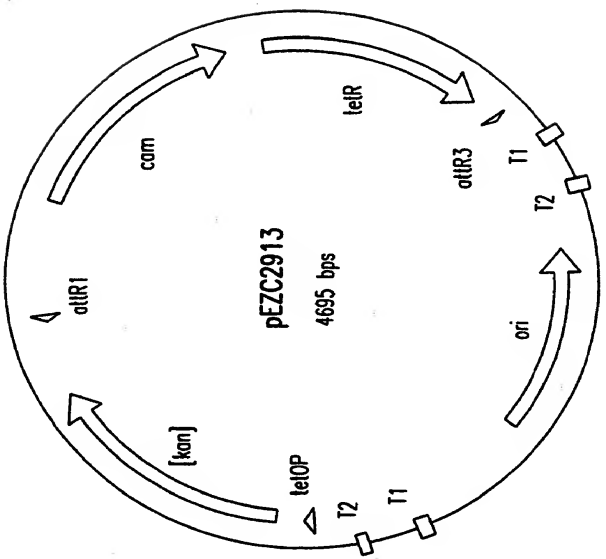
pEZC2913
4695 bps
cam
tetR
attR1
attR3
[kan]
tetOP
ori
T1
T2
T1
T2

FIG.7B

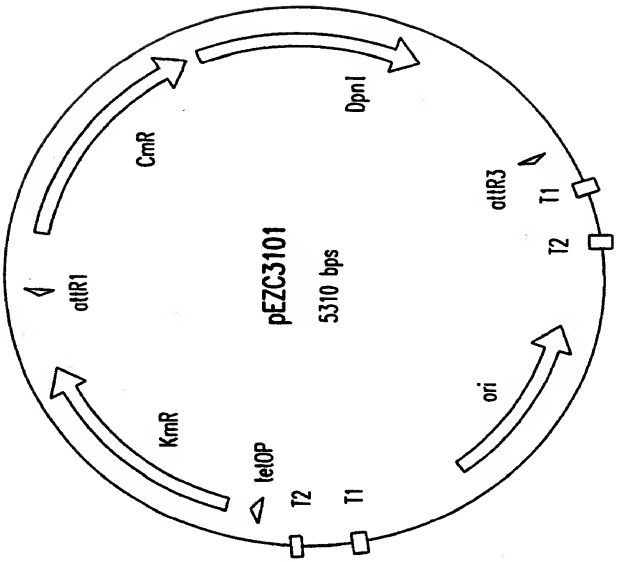
pEZC3101
5310 bps
CmR
attR1
KmR
tetOP
T2
T1
ori
attR3
T1
T2
DpnI

FIG.7C

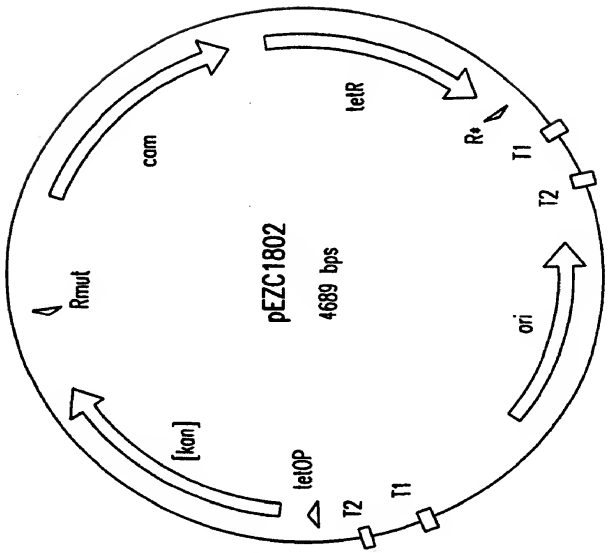

FIG.7D

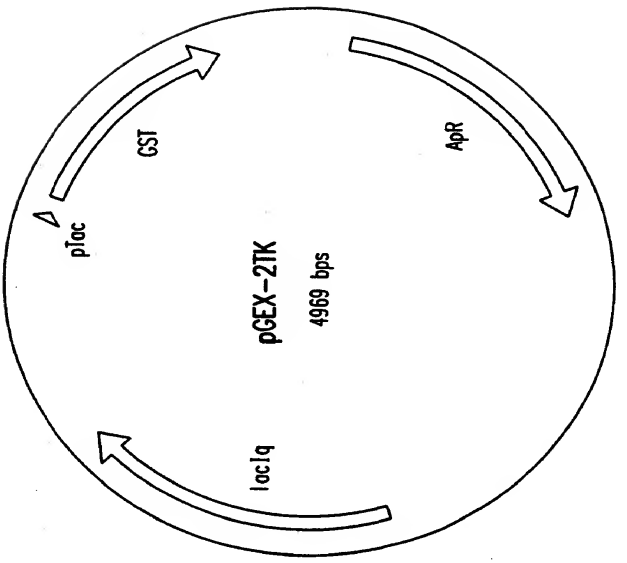

FIG.8A

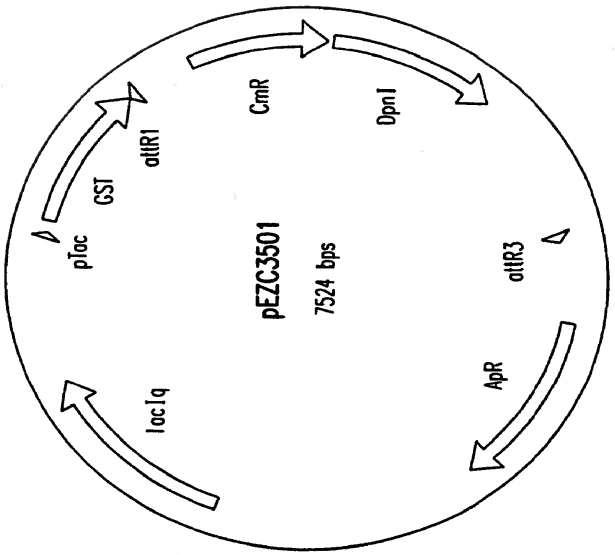

FIG.8B

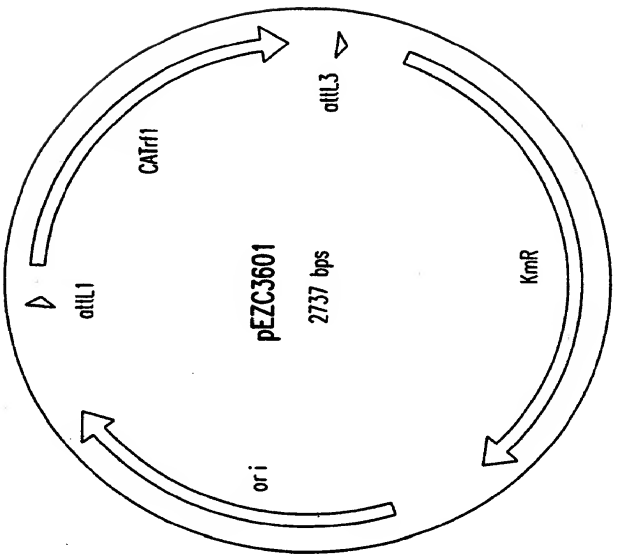

FIG.8C

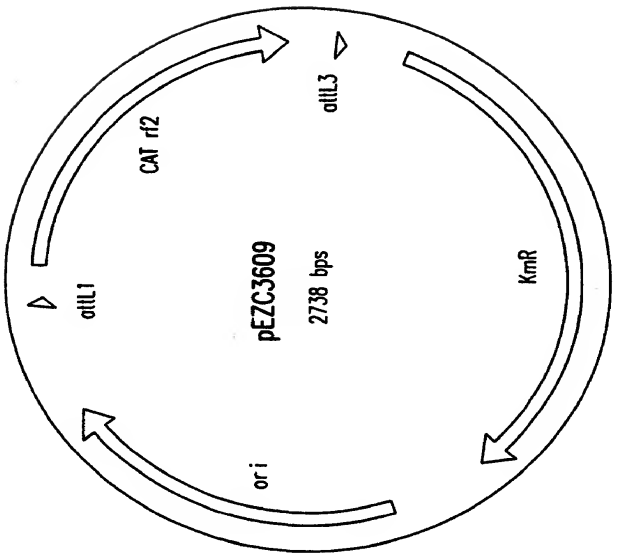

FIG.8D

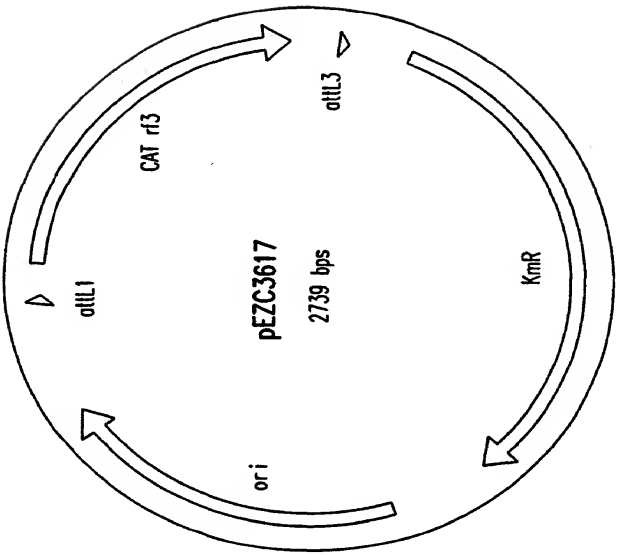

FIG.8E

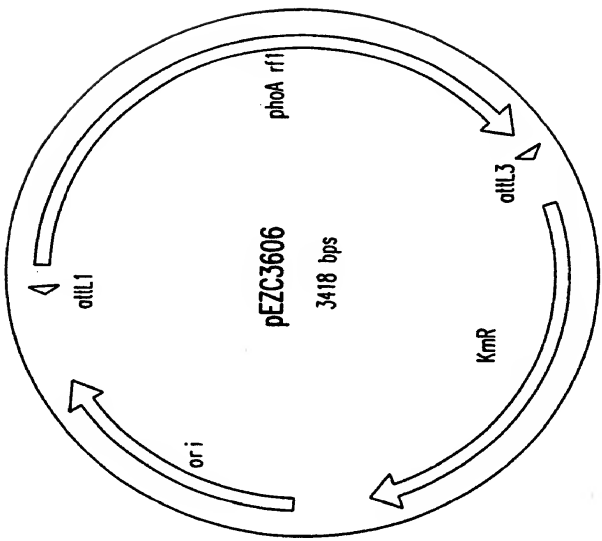

FIG.8F

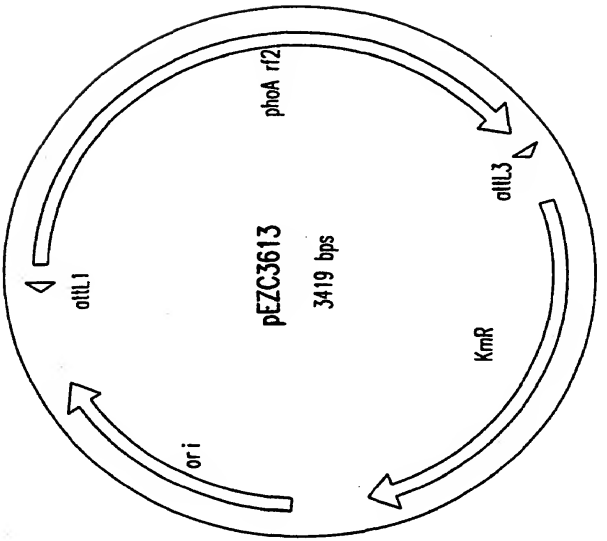

FIG.8G

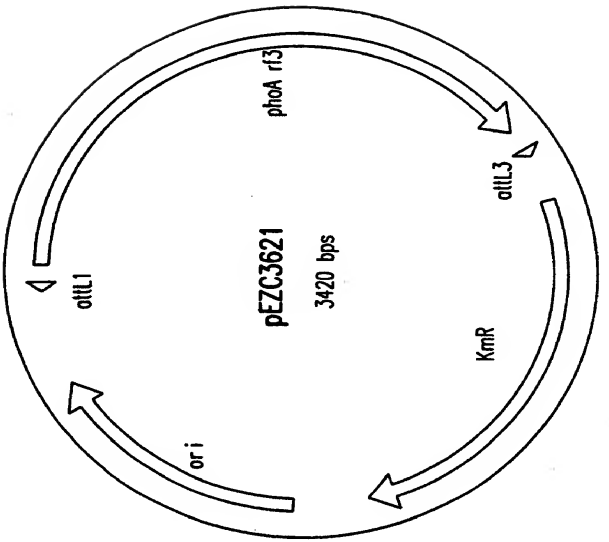

FIG.8H

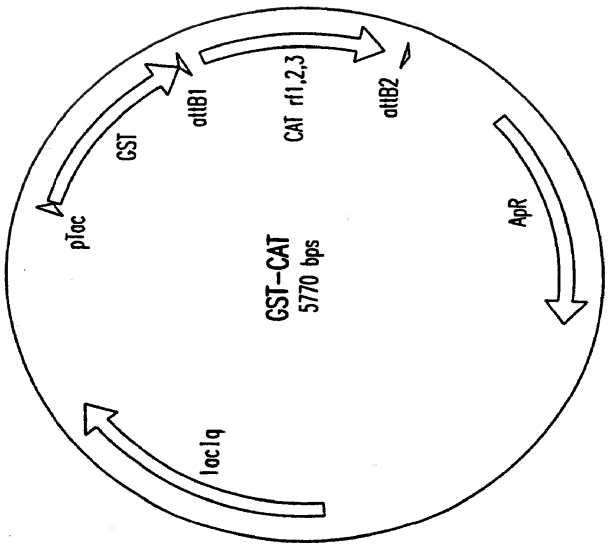

FIG.8I

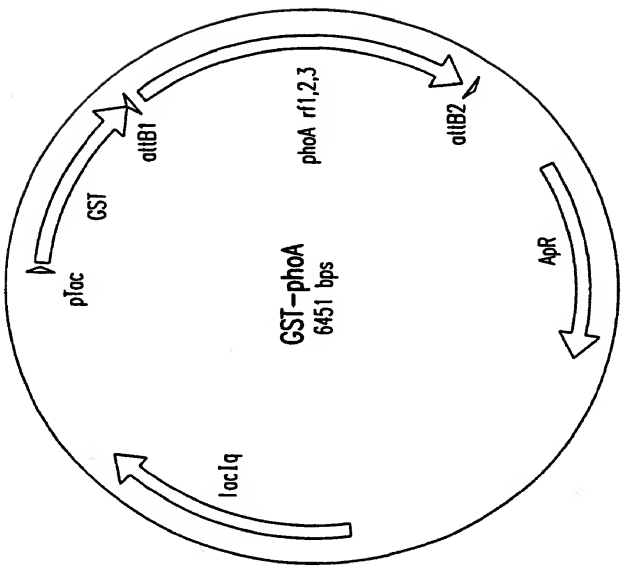

FIG.8J

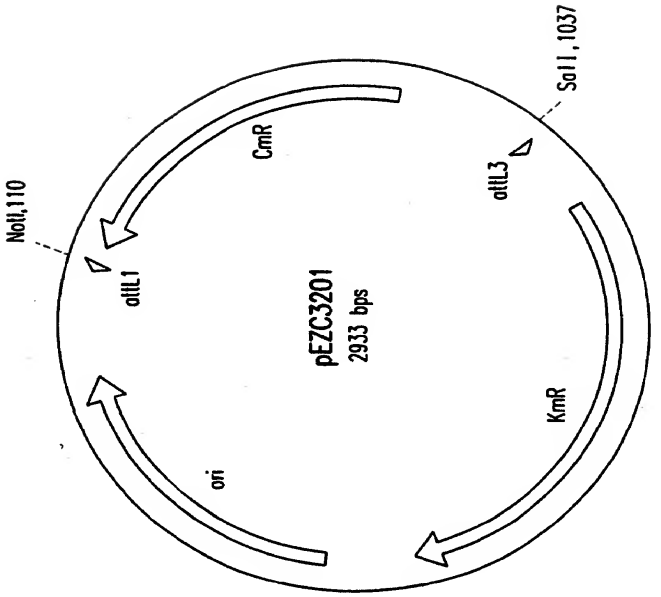

FIG. 8K